THE ROLE OF PHYSIOLOGICAL ELEVATIONS OF GLUCAGON-LIKE

PEPTIDE-ONE IN GLUCOSE REGULATION IN THE DOG IN VIVO

By

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To my family, Mom, Dad, Koller, & Marie

For guiding me to be who I am today,

And for my husband, Matthew,

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CHAPTER I

INTRODUCTION

More than 100 years ago, it was suggested that pancreatic secretion is influenced by "a hormone or secretin yielded by the duodenal mucous membrane" (1). Minkowski and Mering had already shown, in 1890, that internal pancreas secretion is essential for normal carbohydrate homeostasis (1). Not only was it proposed that gut hormones played a role in regulating normal glucose homeostasis, but it was suggested that diabetes may be induced by the "functional disturbance occasioned by the absence of such an intestinal excitant" (1). In 1906, Moore, et al., attempted to determine the role of gut factors in regulating internal pancreatic secretion and glucose homeostasis (1). This included the treatment of diabetic patients with duodenal mucous extracts orally, with mixed results (1). Although it is now understood that oral administration would result in digestion of these factors, those early experiments set the stage for further investigation of the role of the gut in glucose homeostasis.

The goal of this thesis is to further explore the role of a specific gut factor, glucagon-like peptide-1 (GLP-1) in glucose homeostasis. This will be done by evaluation of glucose metabolism during either acute physiological elevations of GLP-1 or inhibition of its action in the conscious dog *in vivo*. Direct and indirect modes of GLP-1 action on liver, muscle, and fat will be considered and discussed.

The Incretin Concept

When healthy individuals receive an oral glucose load, glucose is absorbed from the gut, and circulating glucose levels increase. This increase is sensed by the β-cell of the endocrine pancreas, which initiates an increase in insulin secretion. This increase in insulin secretion results in arterial plasma levels of the hormone that are approximately twice those achieved after an intravenous glucose infusion given at a rate chosen to match glucose levels seen postprandially (2). This augmentation of insulin release is known as the "incretin effect".

Gut Hormones Influence the Endocrine and Exocrine Pancreas

In 1901, work done by Pawlow showed that there is an increase in pancreatic secretion (later to be classified as endocrine secretion) when chyme is released into the duodenum (3). To determine the physiological mechanisms important for this response, Bayliss and Starling, conducted "the crucial experiment" which found that introduction of acid into a denervated jejunum of an anaesthetized dog, increased pancreatic (endocrine) secretion (3). The idea that messages could be sent throughout the body by means other than nerves was a turning point in physiology, leading to the introduction of the concept of a "hormone" (4).

The influence of gut hormones on pancreatic secretion to specifically regulate glucose homeostasis was supported by more conclusive evidence provided several decades later in 1929, by Zunz and La Barre (4). They conducted experiments in which the pancreatic vein of a dog was drained into the jugular vein of a second dog (4). While injection of gut extracts into the former dog only slightly lowered its blood glucose, the

latter dog experienced a significant decrease in blood glucose (4). Zunz and La Barre concluded from their experiments that gut extracts lowered blood glucose levels by stimulating the endocrine pancreas to secrete insulin (4). This provided critical evidence supporting a role for gut hormones in regulating endocrine secretion of the pancreas.

This experiment was followed by further work by La Barre and Still in 1929, which indicated that the crude gut extracts could be purified into two separate components (5). The first factor, which, when injected, induced pancreatic secretion without changing glycemia (5) became known as "excretin", because infusion resulted in "external" pancreatic secretion into the pancreatic duct (6), and what is now known as the exocrine pancreas. The other factor decreased blood sugar (5). This fraction became known as the "incretin," due to its ability to increase "internal" secretion into the pancreatic vein (6), later recognized as the endocrine pancreas, including insulin.

Oral versus Intravenous Glucose Delivery

The physiological importance of incretins was not understood until the mid-1960s, after the development of an immunoassay for insulin by Morgan & Lazarow (7). After this landmark achievement, it was discovered that normal weight humans given an oral glucose load had plasma insulin levels approximately twice those seen when the same group received an intravenous glucose infusion to match the blood glucose profile seen after the oral glucose load (2; 8). At that point the physiological mechanism was unknown, however this phenomena was named the "incretin effect", due to the yet to be defined factors released from the gut resulting in an increase in insulin release.

The Entero-Insular axis

By the late 1960s the phenomenon of gut induced insulin secretion had been termed the "entero-insular axis" (9). This concept described three ways that nutrients in gut induce changes in the pancreas: 1) direct absorption of the nutrients into the blood stream and delivery to the pancreas; 2) neural transmission from the gut to the pancreas; 3) endocrine transmission induced by hormones released from the gut into the circulation (6). (Figure 1.1) Signals from the gut to the pancreas can involve any of these components, either alone or in combination.

After consumption of an orally delivered meal, nutrients are absorbed from the gut and enter the hepatic portal vein followed by entry into the peripheral circulation. As nutrient concentrations in blood increase, direct contact with the islet is increased. Glucose (10) and amino acids (11) increase ATP production by the β-cell, which induces insulin secretion directly. Under normal conditions, free fatty acids increase glucose stimulated insulin secretion, however, excess free fatty acids in the peripheral circulation result in a decrease in glucose stimulated insulin secretion (12). Therefore, nutrients absorbed in the gut can regulate insulin secretion in both a positive and negative manner.

Neural regulation of the pancreas is supported by a vast innervation which has been shown to regulate both its endocrine and exocrine function (13). The islets themselves are surrounded by many nerve fibers, with α-, β-, and δ-cells all innervated by both the sympathetic and the parasympathetic nervous systems (14). Nerves in the gastrointestinal system, referred to as the enteric nervous system, include more that 100 million neurons (15). When a nutrient load reaches the gut, enteric neural signaling is initiated with afferent signals traveling through the vagus either directly to the pancreas
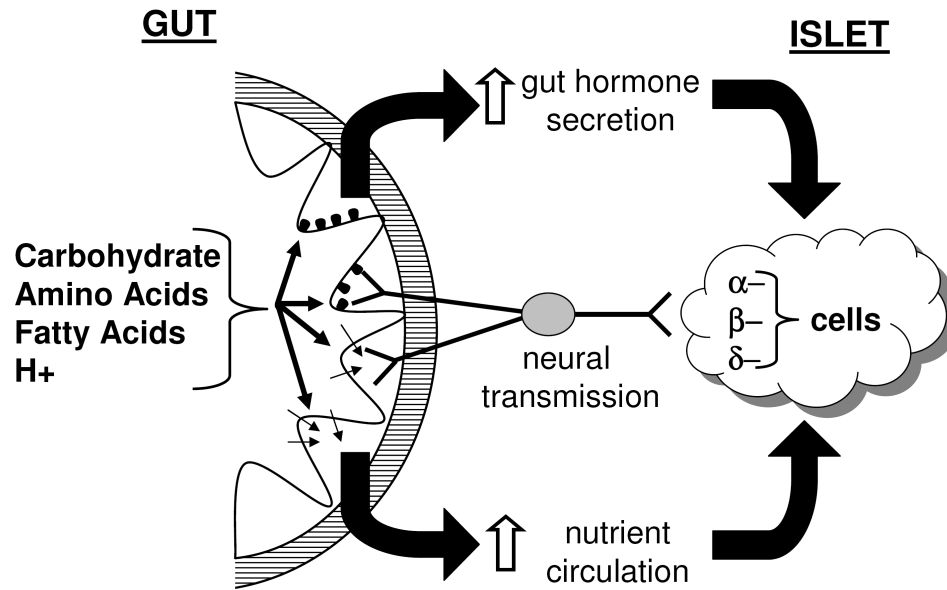
Figure 1.1 The enter-insular axis. Signals to the pancreas in response to nutrient load in the gut. Adapted from Ref. (6).

or to the central nervous system. The latter then alters efferent discharges to the pancreas (15).

The entero-insular axis relies heavily on endocrine transmission from the gut to the pancreas. This transmission occurs by secretion of gut hormones into the circulation, so that they can directly interact with receptors in the pancreas, or by interaction with a receptor at a tissue located closer to the site of secretion, by which it can initiate a neural signal to induce an indirect effect at the β-cell. Gut hormones, secreted in response to a nutrient load, that initiate changes in insulin secretion and glucose homeostasis will be the focus of the remainder of this thesis.

Defining Incretin Hormones

The definition of an incretin hormone as defined by Creutzfeldt involves the following:

1) The hormone must be released from gut endocrine cells after ingestion of nutrients, especially of glucose.
2) The circulating hormone must stimulate insulin secretion in a concentration which is easily achieved after ingestion of a nutrient.
3) The hormone releases insulin only at elevated glucose levels (4).

There are identified incretin hormones: GIP and GLP-1. It has been suggested that the incretin effect, which has been shown to increase insulin secretion by approximately fifty-percent, is due to increases in that these two incretin hormones, although the majority of this work has been done in the mouse (2; 16).

Glucose Dependent Insulinotropic Polypeptide

In 1969, it was discovered that "gastric inhibitory polypeptide," or GIP, isolated from porcine intestine, decreases acid secretion when given in large quantities to dogs (17). It was later determined that GIP induced glucose-dependent insulin secretion at physiological levels, resulting in a re-naming to "glucose-dependent insulinotropic polypeptide" (17). Due to the fact that lower levels of GIP induce its incretin effect, as opposed to GIP's effect on gastric inhibition, it is believed that the physiological importance of the peptide is its ability to induce glucose mediated insulin secretion (17).

GIP is a 42 amino acid peptide (18) secreted by the K cells of the upper small intestine (19; 20) in response to absorption of carbohydrate and fat (21). Inhibitors of absorption block this effect (6). The GIP receptor (GIPR) is member of the vasoactive intestinal peptide (VIP)/glucagon/secretin receptor family of G-protein coupled receptors. When ligand binds it, the adenylyl cyclase signaling cascade is activated (22). GIPR are located in pancreatic islets, adipose tissue, and brain (22). Although GIPR $^{-/-}$ mice exhibit normal weight, fasting glucose levels, and glycemic response to an intra-peritoneal glucose tolerance test, glucose levels after an oral glucose tolerance test (OGTT) were significantly greater in the GIPR $^{-/-}$ than the wild-type (23). The higher glucose levels in the GIPR $^{-/-}$ mice in response to the OGTT were associated with significantly lower plasma insulin levels (23). The decrease in insulin was not due to a defect at the β-cell, because isolated islets from the GIPR $^{-/-}$ mice remained glucose competent; therefore, the impaired glucose tolerance was due to a disruption of the entero-insular axis (23).

This indicates that GIP signaling is required for normal incretin response to oral glucose delivery, reaffirming its classification as an incretin hormone. Although GIP

induces insulin secretion, it was determined that GIP did not account for the full incretin effect observed after oral nutrient delivery, and that another hormone must contribute to the remainder of the effect (4).

Preproglucagon

During the analysis of preproglucagon, it was determined that peptides other than glucagon were coded on the gene, and that these other peptides also influence glucose homeostasis. Preproglucagon is expressed in the α-cell of the pancreas, the L cell in the gut, and the brain. Preproglucagon is translated into the proglucagon amino acid sequence at all of these sites; however, due to different processing enzymes in each of the cell types, different peptides are produced in each location (24). (Figure 1.2) These processing enzymes are serine proteases known as proprotein convertases (PC) which exist in the PC1/3 form in the gut and brain, while PC2 is found in the pancreas (24). Post-translational modification by PC2 results in glucagon as the only active peptide product (25; 26). Inactive products include: glicentin-related pancreatic peptide (GRPP), intervening peptide-1 (IP-1), and the major proglucagon fragment (25; 26). In the presence of PC1/3, glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), two intervening peptides (IP-1, IP-2), oxyntomodulin, and glicentin are the peptides produced (27; 28). (Figure 1.2)

Proglucagon

GRPP	Glucagon	IP-1	**GLP-1**	IP-2	GLP-2

Pancreas (PC2)

major proglucagon fragment

GRPP	Glucagon	IP-1	**GLP-1**	IP-2	GLP-2

Gut & Brain (PC1/3)

Glicentin

GRPP	Glucagon	IP-1	**GLP-1**	IP-2	GLP-2

Oxyntomodulin
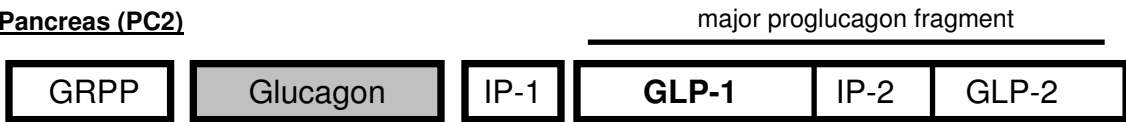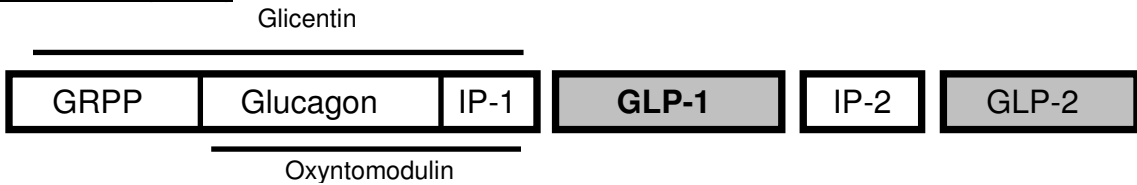
Figure 1.2 Posttranslational modification of proglucagon. Proprotein convertase (PC) 2 is produced in the pancreas, with glucagon resulting as the active petide. In the gut and brain, PC1/3 activity results in GLP-1 and GLP-1 as the major active fragments. GRPP: Glicentin related pancreatic polypeptide; IP; intervening peptide. Adapted from Ref. (24).

Glicentin stimulates gut growth (28), but does not alter insulin section (29). Oxyntomodulin does induce insulin secretion; however, it circulates at such low concentrations, it is unlikely that it contributes to the incretin effect (29). Most evidence suggests that its physiological role is stimulating gastric acid secretion and decreasing food intake (28). GLP-2, although released in response to a nutrient load, does not contribute to the incretin effect, with its primary effects being inhibition of gastrointestinal motility and gastric acid secretion, in addition to protective and regenerative effects in the bowel (28). However, it was determined that the preproglucagon product, GLP-1, does induce insulin secretion at physiological levels.

Glucagon-Like Peptide-1

GLP-1 induces insulin secretion, despite its ~50% amino acid homology with glucagon (30). This peptide has the same amino acid sequence in all mammals (31). In response to an oral nutrient load, GLP-1 is secreted from the L cells in the gut. These cells are referred to as open-type endocrine cells, because the apical membrane has microvilli reaching the intestinal lumen and the base has a large supply of endocrine granules near the basal lamina (26). The L cells secrete two active forms of GLP-1: GLP-1(7-36) amide and glycine-extended GLP-1 (7-37). In the blood GLP-1(7-36) amide represents approximately 75% of active GLP-1, while GLP-1(7-37) is the remaining 25% (24). These two forms of GLP-1 have indistinguishable actions at the endocrine pancreas and identical clearance rates (32).

GLP-1 Secretion

Secretion of GLP-1 is induced by oral delivery of nutrients, specifically carbohydrates and lipids. Absorption of the nutrients is required to induce secretion (33). Postprandial peripheral GLP-1 levels increase rapidly, peaking approximately 15-30 minutes after a meal, with an additional secondary rise occurring at about 90 minutes (34). GLP-1 levels remain elevated for at least 3 hours after oral nutrient delivery in humans (34; 35).

Originally, it was thought that the first peak in GLP-1 level was due to hormonal regulation or neural firing initiated in the upper gut after a meal (36; 37), because it did not appear as though L cells were present in the proximal area of the gut (38). In rodents, GIP secreted from K cells in the upper gut upon contact with nutrients induces GLP-1 secretion either though endocrine or neural pathways (37; 39), however this effect is not observed in humans (40). Endocrine GIP mediated GLP-1 secretion occurs when GIP interacts with its G-protein coupled receptor on the L cell membrane. This increases activation of adenylyl cyclase (AC), which increases cAMP and activates protein kinase A (PKA), resulting in GLP-1 secretion via granule exocytosis (39).

Evidence for neural simulation of GLP-1 secretion has been obtained in vitro, with human and rodent L cells secreting GLP-1 in response to acetylcholine acting via a muscarinic receptor 1(39). When rodents receive a muscarinic receptor antagonist *in vivo*, there is no GLP-1 secretion in response to intraduodenal nutrient delivery (41). It has also been shown that the neuropeptide, gastrin-releasing peptide (GRP), released from neurons in the enteric nervous system activates its G-protein coupled receptor to induce GLP-1 secretion (42). Both acetylcholine and GRP induce GLP-1 secretion by

activating phospholipase C (PLC), which stimulates protein kinase C (PKC), resulting in granule exocytosis (39). Therefore, it is clear that neural signaling can contribute to the secretion of GLP-1; however, its exact role in GLP-1 secretion has now become somewhat controversial due to the fact that recent reports indicate that GLP-1 immunoreactive cells have been detected in the duodenum (43). This new finding would suggest that L cells in the upper gut have direct contact with nutrients soon after consumption, resulting in the early peak in GLP-1 level; therefore, the early secretion of GLP-1 may be less reliant on neural signaling than once thought.

Direct contact of nutrients with the L cell is the accepted explanation for the peak in GLP-1 levels that occurs approximately 90 minutes after a meal. Evidence both *in vivo* (37) and *in vitro* (44-46) shows that GLP-1 secretion is induced in a dose dependent manner when L cells are directly exposed to carbohydrate or lipid. Some suggest that fat is more likely to reach the distal gut, and therefore is responsible for the second postprandial peak in GLP-1 level after a mixed meal (39); however, when our lab administered an oral glucose tolerance test (OGTT) in the dog, the second peak was still observed (47). In addition, glucose infusion, either peripherally (48) or intraportally (49), does not induce GLP-1 secretion, indicating that glucose must have direct contact with the luminal membrane of the L cell to induce GLP-1 secretion.

The L cell is directly stimulated by glucose via sodium glucose transporters (SGLT) 1/3 or glucose transporter (GLUT) 1/5, resulting in increased ATP levels, and subsequent closure of K_{ATP} channels (46). An increase in intracellular K+, in combination with Na+ from the SGLT, depolarizes the L cell, resulting in an opening of voltage gated

calcium channels (39; 50). Increased intracellular calcium levels results in GLP-1 secretion (50).

Monounsaturated fatty acids bind to the receptor GPR120 on the membrane of the L cell (39). It has been shown that when GPR120 is activated, there is an increase in intracellular calcium levels and p44/42 MAPK phosphorylation (51); however when these pathways are both blocked, fatty acids are still able to induce GLP-1 secretion (39; 51). This leaves the mechanism of fatty acid initiated GLP-1 secretion somewhat unclear.

Upon release by the L cell, GLP-1 enters both the intestinal lymph system and portal circulation, with total levels of GLP-1 being approximately 5-6 times greater in the former than the latter (52). GLP-1 secretion, whether in response to GIP, acetylcholine, GRP or a meal, is inhibited by somatostatin (39; 53-55).

Degradation of Incretin Hormones

Dipeptidyl peptidase-4 (DPP-IV) is responsible for the degradation of incretin hormones (56). Also known as the T-cell antigen CD26, it exists in two forms: membrane bound and free in plasma (18; 57). The membrane bound form of DPP-IV is found throughout the vasculature, including that in the kidney and the brush-border membrane of the gut (57). The major site of degradation of incretins by DPP-IV is close to the site of their secretion in the gut brush-border membrane. It has been shown that approximately half of active secreted GLP-1 is degraded by DPP-IV located in the brush border epithelium and the gut capillary endothelium before it reaches the vasculature (58). Once released into the circulation, the intact forms of GIP and GLP-1 are both quickly degraded with half lives of 5 and 1-2 minutes, respectively (59; 60).

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DPP-IV cleaves two N-terminal amino acids from peptides in which the next to the last amino acid is a proline or alanine, as is the case for GIP (1-42), GLP-1(7-36) amide, and GLP-1(7-37). The receptors for both GIP and GLP-1 interact with the N-terminus of the peptide to initiate their respective signaling cascades; therefore, cleavage results in dramatically different interaction at the receptor (61; 62). After exposure of GIP or GLP-1 to DPP-IV, the resulting metabolites GIP (3-42) and GLP-1 (9-36) amide, do not contribute to the incretin effect (63; 64); however, it is still unclear if these metabolites are completely inert. In a small number of studies, infusion of GLP-1 (9-36) amide has been shown to slightly decrease blood glucose via a mechanism independent from insulin secretion and gastric emptying (64; 65); however, the majority of work shows that this metabolite is inert (66-68).

It was once thought that DPP-IV degradation was occurring largely in the kidney. This changed when antibodies were developed for both the active and degraded forms of the incretin peptides. It was determined that the kidneys are not a major site of degradation by DPP-IV, but rather a site of clearance for GIP, GLP-1, and their metabolites (57; 69). The kidneys eliminate ~ 10-20% of the active forms of the peptides (69), which is greater than the rate of glomerular filtration alone, indicating that another mechanism is involved (57). It has been suggested that a portion of the elimination of GLP-1 and GIP occurring at the kidney may be due to degradation by neutral endopeptidase 24.11 (NEP 24.11), which exists in high quantities in the kidney. NEP 24.11 has been shown to degrade GLP-1 and GIP *in vitro* (57). As stated earlier, it is now clear that the majority of degradation of endogenously released GLP-1 occurs by DPP-IV in the gut (58).

Incretin Hormones in Type 2 Diabetes

Type 2 diabetes occurs when the pancreatic β-cells are no longer able to produce enough insulin to compensate for the whole body insulin resistance. It diabetic humans, the incretin effect is significantly decreased relative to that in normal individuals (2). (Figure 1.3) There are several reasons relating directly to incretin hormone secretion and degradation that could explain a decreased incretin effect in those with type 2 diabetes. They are: 1) decreased incretin peptide levels, 2) a decrease in incretin peptide sensitivity at the β-cell, or 3) postprandial glucose levels in type 2 diabetic patients are greater than the stimulus needed for maximal for insulin secretion, resulting in the inability of incretins to further increase insulin release. It is likely that a combination of these contribute to decreased postprandial insulin secretion in those with type 2 diabetes.

A decrease in incretin peptide level could be the result of either an increase in degradation, or a decrease in production. The half life of both GIP and GLP-1 are similar in healthy humans and those with type 2 diabetes, indicating that the degradation of incretin hormones is not altered with the onset of diabetes (59; 60). Incretin hormone production in human patients with type 2 diabetes is somewhat controversial. Early reports indicated that after oral nutrient delivery, GIP levels were similar in type 2 diabetic patients and healthy humans, while GLP-1 was significantly reduced in diabetic patients (70; 71). Although decreased GLP-1 level in type 2 diabetic patients became an accepted explanation for a decrease in the incretin effect, the development of more sophisticated assays, including those that could differentiate between active and inactive forms of GLP-1, resulted in new findings. The more recent data indicated that active and inactive GLP-1 levels could actually be greater in type 2 diabetic patients in response
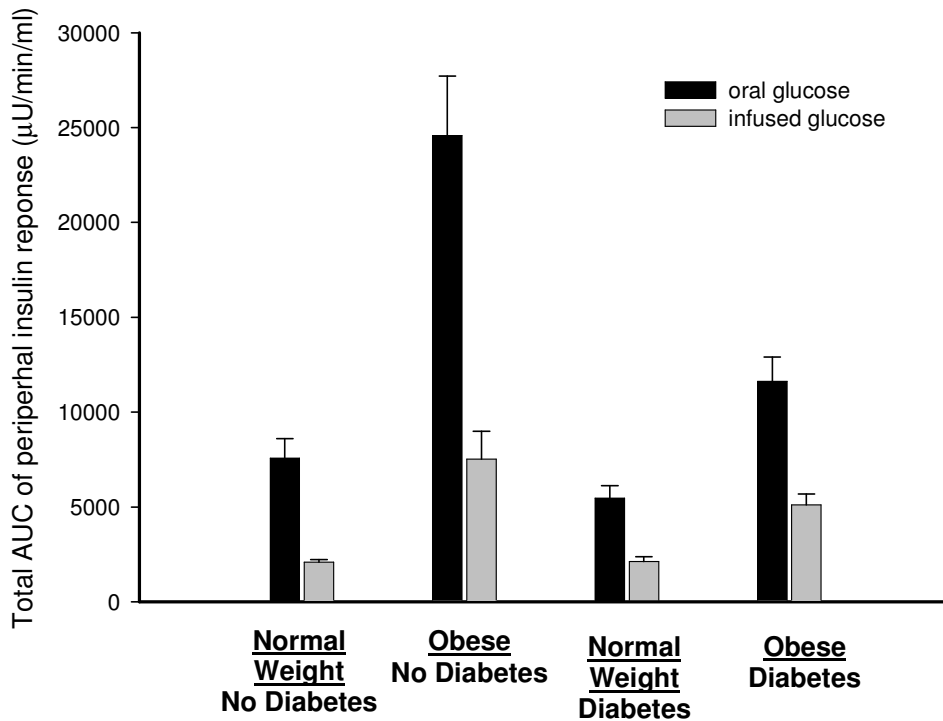
Figure 1.3 The incretin effect in normal weight and obese humans with or without diabetes. Despite greater plasma glucose levels in response to an oral glucose load (not shown), insulin secretion in response was dramatically decreased in those with diabetes, as shown here with total AUC of insulin peripheral insulin profile. When glucose was infused to match the respective peripheral glucose profile of the oral glucose load, insulin secretion was decreased in all groups. Adapted from Ref. (2)

to oral nutrient delivery (43), but this may be in response to greater glycemic levels. In addition, it has been recently shown that DPP-IV levels are decreased in type 2 diabetes, resulting in decreased DPP-IV activity and reduced GLP-1 degradation (72). Overall, there is still much debate about postprandial GLP-1 level in those with type 2 diabetes.

Decreased sensitivity of the β-cell to incretin peptides would also result in a decrease in the incretin effect. In humans with type 2 diabetes, an infusion of GIP to create a physiological increase of the peptide in the periphery in the presence of a hyperglycemic clamp did not increase insulin levels compared to hyperglycemia alone (40). When the GIP infusion rate was doubled to create super-physiological levels in the periphery there was only a slight increase in insulin secretion when compared to hyperglycemia alone (40). The response was markedly decreased from the response observed in normal subjects (40). However, when humans with type 2 diabetes underwent a hyperglycemic clamp and receive a GLP-1 infusion to increase circulating levels to those observed after a meal, insulin secretion was increased when compared to a hyperglycemic clamp alone (40). The insulin response to the hyperglycemia and GLP-1 in those with type 2 diabetes is just slightly less than those observed in healthy subjects infused with the same rate of GLP-1 in the presence of a hyperglycemic clamp (40). Therefore, in humans with type 2 diabetes, resistance of the β-cell to GIP contributes to a decreased incretin effect, but sensitivity to GLP-1 largely remains intact.

Although type 2 diabetes ultimately results in β-cell failure, it has been shown that postprandial glucose levels do not promote maximal insulin secretion, and when peripheral glucose levels are increased to levels greater than found after a meal, insulin secretion is increased in those with the disease (73). In addition, GLP-1 increases insulin

secretion under hyperglycemic conditions in patients with type 2 diabetes, and GLP-1, at both physiologic and super-physiologic levels, can increase insulin secretion over a vast range of hyperglycemia (40; 73). This is evidence that insulin secretion has not reached a maximum rate in the presence of postprandial glycemia in those with type 2 diabetes, per se; however, damage sustained to the β-cell over the progression of developing diabetes may contribute to the decreased incretin effect.

To take advantage of the maintained sensitivity of the β-cell to GLP-1 in diabetic patients that maintain some β-cell function, several novel diabetes therapies have been developed. The established therapies fall into two major categories. The first group consists of GLP-1 mimetics or derivatives that are resistant to degradation by DPP-IV. This includes the GLP-1 mimetic exenatide, a commercial preparation of exendin-4, a peptide found in Gila monster saliva. Another derivative is liraglutide, which is formed by fusing of GLP-1 to a fatty acid, which in turn binds to albumin. GLP-1 bound to albumin is resistant to degradation. The second category of potential diabetes therapies are nonpeptide compounds that target DPP-IV by decreasing its production or inhibiting its action. Other potential therapies include methods (i.g. secretagogues or vectors) to induce increased endogenous GLP-1 production via increased expression of PC1/3 or the peptide itself (74; 75).

GLP-1 Receptor

GLP-1R Characteristics

The GLP-1 receptor (GLP-1R) is a classical guanine nucleotide binding protein (G protein)-coupled receptor of the B1 subfamily, which also includes receptors for GIP,

glucagon, and GLP-2 (76). Although B1 subfamily members have high gene homology, binding at physiological levels only occurs among ligands and their respective receptors (76). Human GLP-1R consists of 463 amino acids, which is 90% homologous to the rat GLP-1R (76; 77). GLP-1R has a dissociation constant of 0.3 nM with GLP-1, and 0.1 nM with exendin-4 (76; 78). GLP-1 binds somewhat at extracellular loops, but primarily to the extracellular amino-terminus of the receptor which, at 146 amino acids, is moderately long like other B family receptors (79). The third intracellular loop of the GLP-1R mediates GLP-1 signaling, whereas the first and second intracellular loops discriminates between various G protein isoforms (80). There is evidence that the GLP-1R is coupled to G_s, G_{i1}, G_o, and G_{l1} (80).

Early characterization of the GLP-1R was conducted with radioligand assays, resulting in evidence of a single receptor type in rat insulinoma-derived cells (81; 82). Cloning of the GLP-1R in the islet of the rat resulted in two different RNA spicing variants, but a single established amino acid sequence to compare against all other tissues (83). The tissue distribution of GLP-1R *in vivo* is quite controversial. This is due to the fact that locations of the receptor have been identified in different manners and in different species. Different methods of detection vary from radiolabelling and immunoassays, to changes in neural firing and metabolism. (Table 1.1)

Site of identified GLP-1R	Technique of Identification
Pancreas	-Binding of GLP-1 to α-, β-, δ-cell of rats by radiolabeled assay (84) -GLP-1R protein detected in rat β-cell by Western blot (85) -GLP-1R RNA detection in islets of rats, dogs, and humans by PCR followed by Southern blot of cDNA (86-90) -GLP-1R RNA detected in rat and human by Northern blot (83; 91; 92) -Increased insulin and somatostatin secretion, accompanied by decreased glucagon secretion in canine and rat pancreases perfused with GLP-1 (93)
Brain & Nervous System	-Binding of GLP-1 in rat brain by radiolabeled assay (94) -GLP-1R RNA in rat CNS by in situ hybridization (95) -Detection of GLP-1R RNA in rat brain by Northern blot (91) -GLP-1R RNA detected in rat and human brain by PCR/Southern blot (89; 90)
Hepatic Portal Vein	-GLP-1R RNA identified in nerve terminals within the hepatic portal vein of rat by PCR/Southern blot (87) -Increased afferent neural discharges from the region when rats given intraportal GLP-1 bolus (96)
Liver	-Binding of GLP-1 in rats by radiolabeled assay (97) -GLP-1R RNA identified in rat by Northern blot (91) -GLP-1R RNA identified in rat by PCR/Southern blot (88) -GLP-1 decreases human endogenous glucose production, independently of changes in pancreatic hormones (98) -GLP-1 infusion increases canine hepatic glucose uptake (47; 48) -GLP-1 increases glycogen storage in rat hepatocytes (99)
Adipose	-Binding of GLP-1 to human adipose by radiolabeled assay (100) -GLP-1R RNA identified in rat and canine by PCR/Southern blot (86; 88) -GLP-1 increases insulin mediated glucose uptake in adipocyte cell line (88) -GLP-1 increases nonhepatic glucose uptake in canine (48)
Skeletal Muscle	-Binding of GLP-1 to rat skeletal muscle (101) -GLP-1R RNA detected in rat by Northern blot (91) -GLP-1R RNA detected in rat and canine by PCR/Southern blot (86; 88; 90) -GLP-1 increases nonhepatic glucose uptake in canine (48)
Heart	-GLP-1R RNA detected in rat and human by PCR/Southern blot (88-90)
Kidney	-GLP-1R RNA detected in rat by Northern blot (83; 91) -GLP-1R RNA detected in rat and human by PCR/Southern blot (89; 90)
Lung	-GLP-1R RNA detected in rat by Northern blot (83; 91) -GLP-1R RNA detected in human and rat by PCR followed by Southern blot (87; 89; 90)
Stomach	-GLP-1R RNA detected in rat by Northern blot (83) -GLP-1R RNA detected in rat and human by PCR/Southern blot (89; 90)
Intestine	-GLP-1R RNA detected in rat and human by PCR/Southern blot (88; 90)

Table 1.1 Positive identification of GLP-1R locations.

Radiolabeled assays have been used to determine GLP-1 binding to α-, β-, and δ-cells of the islets of Langerhans, the brain, liver, and skeletal muscle of rats, as well as human adipose tissue (84; 94; 97; 100; 101). While Western blots confirmed the presence of GLP-1R on the β-cell, there was no indication of GLP-1R on the α-cell using this technique (85). Northern blot analysis of rat tissue confirmed the presence of GLP-1R RNA in the islet, in addition to the stomach, kidney, and lung, but not in the smooth muscle, spleen, heart, testis, intestine, or colon, with mixed results for rat liver, skeletal muscle, and brain (83; 91). Northern blot analysis of human tissue indicated positive results for the pancreas, but not in lung, smooth muscle, heart, brain, spleen, liver, skeletal muscle, or kidney (92). In situ hybridization has resulted in identification of GLP-1R throughout the central nervous system of the rat (95). Also in the rat, PCR, followed by Southern blot of cDNA, identified GLP-1R in the nodose ganglia and nerve terminals innervating the portal vein, pancreatic islets, lung, skeletal muscle, adipose, intestine, stomach, kidney, brain, and heart, with mixed detection at the liver, and none in the spinal cord (87; 88; 90). This same technique in the dog found GLP-1R located in the pancreas and fat, with much lower levels in skeletal muscle, and no detection in liver (86). GLP-1R RNA has also been detected in human pancreas, lung, brain, stomach, kidney, heart, but not in liver, skeletal muscle, or adipose by PCR followed by Southern blot (89).

There has been suggestion of GLP-1R in some tissues due to effects that result from the presence of GLP-1. Perfused canine and rat pancreases produce increased insulin and somatostatin in the presence of a GLP-1 infusion (93), confirming the presence of a receptor for GLP-1 in the pancreatic islet. Although there is no

confirmation of GLP-1R RNA in the liver, isolated rat hepatocytes have increased

glycogen storage in the presence of GLP-1 (99). In addition, hepatic glucose production

is decreased in humans that receive GLP-1 intravenously, in the presence of a pancreatic

clamp (98). An intraportal bolus of GLP-1 resulted in increased afferent discharges from

the hepatic branch of the vagal nerve in rat (96), confirming the presence of a receptor for

GLP-1 in the hepatic portal vein. Although there have been mixed opinions on whether or

not the GLP-1R is located in insulin sensitive tissue (liver, muscle, and fat), GLP-1 does

increase glucose utilization in these tissues, when pancreatic hormone levels are held

constant (47; 48; 86; 88).

Discrepancies in determining GLP-1R tissue distribution could be explained by

the use of different methods and various species used to identify the location of the

distribution of GLP-1R; however, there are other possible explanations. It has been

suggested that an as yet to be identified receptor, other than the classical GLP-1R, exists

and that GLP-1 may be mediating a portion of its effects in some tissues via this other

receptor (102). This is also supported by the fact that even though only one receptor has

been identified, it has been shown that alternate gene splicing can result in two separate

transcripts (83; 91). Others have suggested the GLP-1R may be present in very low

abundance in some tissue, such that it is not detected when compared to the high levels of

the receptor in the islet (92). Another possibility is that the GLP-1R is not uniformly

expressed throughout certain tissues (92). Additionally, due to the presence of GLP-1 in

the central nervous system, it is possible that GLP-1 has indirect effects intiated by

interaction with its receptors at a nerve terminus in one location, which results in effects

that are manifested at another tissue. Therefore, it appears as though GLP-1 could

mediate its actions by direct interaction with its receptor and through indirect pathways via neural mediation.

GLP-1 Signal Transduction

The presence of GLP-1R in the β-cell is fully accepted; therefore, the pathway of GLP-1 signaling has been investigated primarily in the pancreas. Remarks in reference to GLP-1 signaling have been determined in insulin producing cells, unless otherwise specifically noted.

Although it has been shown that GLP-1R activates various G-protein isoforms (80), in the β-cell, GLP-1R primarily activates $G_s\alpha$, which in turn activates adenylyl cyclase (AC) (103). Increased activation of AC results in production of cAMP, which is the main mediator of GLP-1 effects (104; 105). Negative regulation of cAMP occurs in the presence of cyclic nucleotide phosphodiesterases (PDE) (106). Although less frequently, GLP-1 does initiate cAMP-independent signaling which is regulated by phosphatidylinositol-3 (PI3) kinase. Even though this pathway is much less defined, it is likely that PI3 kinase is activated by the $G_{\beta\gamma}$ subunit of the GLP-1R (103). Further detail of GLP-1R signal transduction is given in conjunction with the effects of GLP-1.

Elimination or Blocking the GLP-1R

GLP-1 is translated from the preproglucagon gene, which also includes other peptides that mediate glucose homeostasis; therefore, a knockout model of some or the entire preproglucagon gene may result in changes in glucose metabolism that do not truly reflect the lack of the GLP-1 protein. To determine the specific physiological effects of GLP-1, null mutation of the GLP-1R was created (107). Fasting glucose levels of GLP-

1R$^{-/-}$ mice are similar to those of wild type animals; however, in response to an oral

glucose load, peripheral glycemia is significantly greater in the GLP-1R$^{-/-}$ mice, with no

compensatory increase in insulin secretion (107). The true contribution of GLP-1 to

regulating glucose homeostasis may, however, actually be much greater than exhibited by

the glycemic response of the GLP-1R$^{-/-}$ mice. These animals exhibit significantly greater

circulating GIP levels, in addition to much greater GIP sensitivity at the β-cell, which

may compensate for the lack of GLP-1 signaling (108).

Not only do GLP-1R$^{-/-}$ mice show increased GIP action (108), but GIPR$^{-/-}$ mice

also have a compensatory increase in GLP-1 sensitivity (16; 109; 110). This makes it

difficult to evaluate the specific contributions of either peptide. To better evaluate the true

physiological contributions of incretin hormones, double incretin knockout (DIRKO)

mice were developed (109). The DIRKO mice have greater peripheral glycemia than

either of the single knockout animals after an oral glucose tolerance test (109). This is

the result of significantly reduced plasma insulin levels, despite the higher glycemia

(109); therefore, the response of the DIRKO mice confirm the combined contribution of

GIP and GLP-1 to the incretin effect, but the individual knockouts do not specifically

define the individual physiological role of either hormone.

There is another approach to determining the physiological effects GLP-1. It is

the use a GLP-1R antagonist, exendin (9-39) (78; 111). Exendin (9-39) is a truncated

form of the previously mentioned exendin-4, and acts in a competitive manner to

specifically block the GLP-1R (78). This provides a tool to clarify the physiological role

of GLP-1 by acutely blocking GLP-1 signaling. This negates the concerns of

compensatory mechanisms that occur with the chronic ablation of GLP-1 signaling observed in the GLP-1R$^{-/-}$ mice.

<u>GLP-1R and Diabetes</u>

It has been determined that in individuals with type 2 diabetes, β-cell sensitivity to GLP-1 is slightly decreased compared to healthy subjects (40). Changes that may occur at the GLP-1R with this disease have yet to be defined. There is no correlation of common allelic variants of the GLP-1R gene with type 2 diabetes (112; 113). In one patient with type 2 diabetes, but not in any control subjects, a specific mutation of the GLP-1R was identified which resulted in a 60-fold decrease in binding affinity for active forms GLP-1, with no difference in binding of either GLP-1 (9-36) or exenatide (9-39) (114). In rats, it has been shown that GLP-1R mRNA levels are decreased in islets in response to hyperglycemia (115). In addition, these levels are also decreased in the brain of the obese Zucker rats (116). This evidence suggests that decreased sensitivity of the β-cell to GLP-1 in type 2 diabetes could be partially attributed to either decreased binding affinity of the GLP-1 to its receptor or decreased GLP-1R expression.

Effects of GLP-1

Although GLP-1 was first identified as an incretin hormone, there is a large body of evidence indicating that GLP-1 acts throughout the body as a glucoregulatory factor (117; 118). The following is a summary of how GLP-1 affects various tissues and organs, including a description of GLP-1 mediated signaling. Direct effects of GLP-1 include actions that are mediated by GLP-1 binding to a receptor at the tissue in which

the effect is observed. These include tissues in which it has been proven or suggested that the GLP-1R is present. Indirect effects are those that are initiated by GLP-1 interaction with its receptor at a site other than which the effect is observed. Indirect effects are the result of paracrine or neural transmission from the site of initiation to the site of the resulting effect. It has been shown that GLP-1 exerts both direct and indirect effects.

<div align="center">Insulin Secretion</div>

The most well defined effect of GLP-1 is glucose-dependent insulin secretion. As mentioned previously, activation of cAMP is the major signaling pathway initiated upon GLP-1 binding with its receptor at the β-cell. Increased levels of cAMP are required for glucose-dependent GLP-1 induced insulin secretion (119). GLP-1 signaling initiates two major downstream pathways of cAMP. The first major regulator is the increased activity of PKA (120); however, when PKA is blocked, insulin secretion stimulated by GLP-1 is only decreased by 50-60%, supporting the fact that a second signaling mechanism is in place (103; 119). The second pathway is mediated by "cAMP regulated guanine nucleotide exchange factors" (cAMPGEFs) also known an "exchange proteins directly activated by cAMP" (Epac) (119; 121). cAMP has a significantly lower affinity for Epac than PKA; therefore, lower levels of cAMP activate PKA, and when PKA signaling is saturated, cAMP continues to increase signal transmission by Epac activation (103; 121).

Glucose uptake by GLUT2 at the β-cell increases the intracellular ATP/ADP ratio (122-124) . Epac augments this increase in the ATP/ADP ratio, via a Ca^{2+} dependent mechanism (125). K_{ATP} channels are closed by this increased ATP/ADP ratio (126). GLP-1 activated PKA increases K_{ATP} channel closure by direct phosphorylation of the

channel (127), and there is some evidence that Epac also augments K_{ATP} channel closure

(128). This results in quicker depolarization of the cell (129), thereby increasing the

intracellular Ca^{2+} content by opening membrane Ca^{2+} channels and causing exocytosis of

Ca^{2+} from stores in the endoplasmic reticulum (ER). Both routes of increased

intracellular Ca^{2+} are augmented by PKA and Epac (125; 130; 131). Increased

intracellular Ca^{2+} results in exocytosis of insulin granules from the plasma membrane.

There is evidence that both PKA and Epac also interact directly at the insulin vesicles to

aid exocytosis (132). GLP-1 induced activation of PKA also results in greater frequency

of insulin granule exocytosis by antagonizing K_v channels (133; 134). K_v channels

oppose the actions of K_{ATP} channels by decreasing intracellular K^+ concentration, which

results in repolarization of the β-cell (133; 134).

GLP-1 also initiates effects at the β-cell which increase the availability of insulin

for secretion. Insulin producing cells have increased intracellular insulin content after

incubation with GLP-1 (135). This effect is mediated by both cAMP/PKA and PI3-

kinase/cAMP-independent pathways (103). GLP-1 increases mRNA levels of

transcription factor PDX-1 and promotes its transport to the nucleus (136-138). Nuclear

PDX-1 increases insulin mRNA levels (139). This has been directly linked to GLP-1

induced increases in intracellular insulin content (104; 135).

GLP-1 also promotes continued activation of PDX-1 by PI3 kinase activation of

PKB. This phosphorylates forkhead transcription factor O1 (FoxO1), which traps it in

the cytoplasm, making it unable to block nuclear PDX-1 action (103; 140). In addition,

GLP-1 activates polypyrimidine tract binding protein 1 (PTB1), which stabilizes insulin

and insulin vesicle protein mRNA, making it less susceptible to degradation (103; 141).

There is also some evidence that GLP-1R activation enhances second phase insulin secretion by shifting less accessible pools of insulin granules closer to the membrane of the cell (130; 142). Combined with the aforementioned effects, this perpetuates GLP-1 enhanced insulin secretion well after the induction of hyperglycemia (40; 73).

There have been additional reports to suggest that indirect mechanisms also mediate GLP-1's effect on insulin secretion. When GLP-1 is infused into the brain of mice, glucose-dependent insulin secretion is increased (143). Others have suggested that a neural circuit exists between GLP-1 receptors in the hepatic portal vein and the pancreas (96). Although it is well documented that GLP-1 does have a direct effect at the pancreas, these studies indicate that elevations of GLP-1 observed after a meal may initiate the incretin response in a variety of ways.

β-cell Mass

Chronic GLP-1R activation in human isolated islets, as well as *in vivo* in rodents results in increased β-cell mass (144-147). This effect has been shown to be independent of both the peripheral glucose level and GLP-1's ability to lower glycemia (147; 148). Although GLP-1 appears to be expressed during embryonic development, and embryonic β-cells do express GLP-1R, it has yet to be determined the extent to which endogenous GLP-1 increases or maintains adult β-cell mass in a healthy physiological setting (149). In addition, it has yet to be fully determined the effects of GLP-1 on β-cell mass in humans. Even so, the ability of GLP-1R activation to result in increased β-cell mass may eventually be used for the development of therapies for improved β-cell mass in both type 1 and 2 diabetes.

Increased β-cell mass occurs by increasing the ratio of β-cell expansion to β-cell depletion. GLP-1 has been shown to increase this ratio by multiple mechanisms, mainly initiated by cAMP/PKA and PI3-kinase/cAMP-independent pathways, which activate PDX-1 (103; 150; 151). PDX-1 not only regulates insulin transcription, but is also a major determinant of β-cell generation and maintenance (152; 153).

Generation of β-cell mass *in vivo* occurs in three ways: 1) neogenesis, which includes differentiation from precursor cells; 2) proliferation by division of existing β-cells; and 3) hypertrophy by enlargement of existing β-cells (154). Established β-cell precursor cells are found in islets and pancreatic ducts *in vivo* (155; 156). In the presence of GLP-1, PDX-1 is up-regulated in precursor cells from both of these regions, resulting in the transformation of these cells into insulin producing cells (157; 158). Stem cells are a potential source of exogenously produced β-cells for those with type 1 or 2 diabetes. GLP-1, in conjunction with additional factors, also converts stem cells into insulin producing cells (159; 160). GLP-1 also promotes proliferation and hypertrophy of established β-cells through PI3 kinase activation of PKB, which deactivates FoxO1, allowing PDX-1 to localize to the nucleus (137; 144; 146; 161; 162).

Depletion of β-cell mass occurs in two ways: cell death, mainly via apoptosis and atrophy of existing β-cells (154). GLP-1 inhibits β-cell apoptosis and promotes β-cell integrity in vitro (145; 163). This has been confirmed *in vivo* in Zucker diabetic fatty (ZDF) rats, which have decreased β-cell mass due to increased apoptosis (164). When ZDF rats are chronically treated with GLP-1, islet mass and β-cell proliferation is significantly increased, while β-cell apoptosis is significantly decreased (165). Taken

together it is clear that the actions of chronic activation of GLP-1R in the pancreas protects and expands β-cell mass.

GLP-1 Regulation of the α-cell

GLP-1 has been shown to decrease inappropriately high glucagon levels in diabetic patients (166). Similar to GLP-1's ability to induce insulin secretion, the effect on glucagon secretion is glucose dependent, meaning that GLP-1 will only block elevated glucagon secretion under conditions of hyper and euglycemia (167). GLP-1 does not seem to have this effect on healthy patients (168). The presence of the GLP-1R on the α-cell is controversial, because it has been identified by some (84), but not others (85). Despite what is observed *in vivo*, intracellular cAMP levels do not change in isolated α-cells in the presence of GLP-1 (85). In addition, isolated α-cells actually increase glucagon secretion in the presence of GLP-1 (169); therefore, what would appear to be a direct effect of GLP-1 on the α-cell may actually be an indirect effect resulting from a direct GLP-1 effect elsewhere in the islet.

One possibility is that the GLP-1 induced decrease in glucagon secretion observed *in vivo* is actually a secondary effect of enhanced insulin secretion. Insulin could be acting in a paracrine manner with its known receptor at the α-cell (170). It has been shown that insulin does directly inhibit glucagon secretion from isolated α-cells (170). *In vivo*, mice that are unable to secrete insulin in response to glucose are unable to suppress glucagon secretion (171; 172). In addition, increased Zn^{2+} in the islet, which is packaged and released with insulin granules, inhibits glucagon secretion (170). Increased insulin secretion associated with GLP-1 may result in increased Zn2+ release, which could be

responsible for decreased glucagon secretion. Some have also suggested that the decrease in glucagon secretion is a result of the direct effect of GLP-1 resulting in an increase in α-cell insulin sensitivity (167).

It has been well documented that the direct effect of GLP-1 on pancreatic δ-cells is increased somatostatin release (173; 174), which could inhibit glucagon secretion in a paracrine fashion. This is supported by studies indicating that GIP does not alter somatostatin release from pancreatic δ-cells or decrease glucagon secretion in humans (103; 173; 175). Due the suggested blood flow in the islet from β- to α- to δ-cell, it seems most likely that the effect of GLP-1 on glucagon secretion *in vivo* is actually an indirect result of GLP-1's direct effects on the β- , and possibly to a lesser effect the δ-cells, in the pancreas.

GLP-1 Regulation at the Liver

The liver is responsible for the disposition of approximately one-third of circulating glucose after an orally delivered moderate glucose load (176). With ~80% of hepatic blood supplied by the hepatic portal vein GLP-1 levels are significantly greater at the liver than in the periphery (47). While liver glucose production and uptake are indirectly regulated by GLP-1 induced changes in pancreatic hormone levels, there is some evidence that GLP-1 can exert a direct effect on the liver, per se (47; 177).

There has been very little evidence that the classical GLP-1R is present in the liver. The use of PCR to detect GLP-1R mRNA has brought about mixed results, with minimal levels at best (86; 88), but it has been shown that radiolabeled GLP-1 does bind

to hepatic membranes (97). This suggests that direct regulation of the liver by GLP-1 resulting in changes in glucose production and utilization is possible (97; 102).

In vivo, a peripheral infusion of GLP-1, to create a physiological increase in GLP-1 levels, suppressed glucose production in humans under euglycemic clamp conditions with pancreatic hormones clamped at basal levels (98). Our lab has shown in dogs that when a physiological or pharmacological increase in plasma GLP-1 is brought about under hyperglycemic-hyperinsulinemic clamp conditions there is an increase in net hepatic glucose uptake (47; 48; 177). This effect occurs whether GLP-1 is administered intraportally or via the hepatic artery (47; 177), suggesting that GLP-1 is mediating this effect directly by activating its receptors at the liver. This effect is small and seen only with high physiological levels of the peptide (177).

In vitro, hepatocytes incubated in GLP-1 increase glucose incorporation into glycogen due to increased glycogen synthase and decreased glycogen phosphorylase activity (178). Changes in enzyme activation are the result of cAMP-independent signaling via PI3 kinase/PKB pathways (179). This is in agreement with the inhibition of glucagon-induced glycogenolysis by GLP-1 (180). When GLP-1 levels are elevated in the brain of mice, insulin mediated hepatic glucose uptake and conversion into glycogen is favored over nonhepatic glucose uptake (143). These results indicate that GLP-1 enhances hepatic glucose uptake and storage by both direct and indirect mechanisms.

<u>Direct Effects of GLP-1 on Adipose and Skeletal Muscle</u>

There is *in vitro* evidence that GLP-1 exerts a direct effect at the adipocyte. GLP-1 binds to the adipocyte membrane (100; 181) and has been shown to increase intracellular cAMP levels in primary adipose culture (182). In 3T3-L1 adipocytes, GLP-1 increases insulin mediated glucose uptake (88). In this cell line, increased insulin mediated glucose uptake in the presence of GLP-1 results in increased lipid synthesis (88). This is in agreement with experiments in primary cultures of adipocytes in which GLP-1 increased insulin dependent incorporation of radiolabeled acetate into fatty acids (183). Although there is agreement between cell line and primary culture in regard to fatty acid synthesis, there are mixed results when evaluating glycogen synthesis, with increases in primary cultured adipocytes (184), and no effect in 3T3-L1 adipocytes (88). These effects are thought to be mediated through a PI3 kinase/MAPK pathway (185), and are not the result of changing the affinity of insulin for its receptor (88).

The effects of GLP-1 on skeletal muscle have also been explored *in vitro*. It has been determined that GLP-1 binds to rat skeletal muscle, but that binding is not accompanied by changes in AC or cAMP (101; 186). Similar results were found in L6 myotubes exposed to GLP-1; however, when these cells were transfected with the identified GLP-1R, they experience a significant increase in intracellular cAMP (187). This suggests that an unidentified receptor for GLP-1 at the muscle may be mediating its effects. There is some evidence that signaling is occurring via PI3 kinase (188-190); however, these studies have all been conducted in the same laboratory, and others have not been able to confirm these results (191). Muscle tissue exposed to physiological levels of GLP-1 exhibits increased glucose uptake and glycogen storage, due to increased

glycogen synthase activity and decreased glycogen phosphorylase activity (178; 186; 192; 193). Other effects of GLP-1 in muscle include increased glucose oxidation and increased lactate production (186; 192; 193).

In vivo studies suggest that GLP-1 serves as an insulin sensitizer throughout the body. GLP-1 has been shown to increase whole body glucose disposal to a greater extent than can be attributed to changes in the insulin level (194). The majority of this increase in uptake has been attributed to increased adipose and skeletal muscle glucose uptake (194). GLP-1 increased whole body glucose uptake in the presence of a hyperglycemic-hyperinsulinemic clamp in depancreatized dogs (86). Our lab has also shown that GLP-1 increases nonhepatic glucose uptake in the presence of GLP-1 under a hyperglycemic-hyperinsulinemic clamp (48). Others have found no such effect (195). The studies referenced here delivered GLP-1 via a peripheral infusion, rather than through an intraportal route, which would be a more physiologically relevant route of delivery. The significance of route of GLP-1 delivery will be discussed further in a later section.

At this point, it is difficult to determine if *in vivo* actions of GLP-1 are the result of direct effects on glucose utilization, or if its role in whole body glucose homeostasis is dependent on its enhancement of insulin action. If a direct effect of GLP-1 does exist, it is small and hard to isolate from the other effects of GLP-1. The effect of GLP-1 on whole body glucose utilization discussed in this section focuses on acute elevation of GLP-1 levels. Chronic elevations of GLP-1 have an effect on body weight, which would also influence glucose utilization. Chronic GLP-1 treatment and body weight will be discussed in a later section.

Cardiovascular Regulation

Changes in circulation play an important role in nutrient delivery, and therefore, the role of GLP-1 in the cardiovascular system may result in changes in glucose utilization. It has been shown that GLP-1 exerts both direct and indirect effects in the cardiovascular system. Pharmacological concentrations of GLP-1 administered intravenously into rats result in significantly increased systolic, diastolic, and mean arterial blood pressure (196; 197). The GLP-1R has been identified in heart tissue and, when activated, the result is increased cAMP levels (198). In addition, GLP-1 decreases pulmonary vascular tone in isolated, perfused lung, indicating a direct effect within the lung (199). Mean arterial blood pressure and heart rate were also increased when GLP-1 was injected into rats via intracerebroventricular administration (197; 200). These effects were most likely initiated at GLP-1R known to exist in the nucleus tractus solitarius, which is a controller of cardiovascular function (199; 201).

Some have suggested that GLP-1 increases glucose uptake by the heart by increasing insulin sensitivity, which in turn improves cardiac function (202; 203). This may explain the benefits observed with chronic GLP-1 treatment of those with impaired cardiac function (204; 205). There is potential for GLP-1 to serve as a therapy to improved cardiovascular function, but its role as a physiological regulator of the cardiovascular system is unresolved. Few *in vivo* studies report indicators of blood flow, making it difficult to address the effects of GLP-1 on cardiac function; however, our lab has reported increases in hepatic and nonhepatic glucose uptake in the presence of physiological GLP-1, without changes in blood flow to the liver (48). This indicates that

GLP-1 effects on whole body glucose utilization are unlikely to be due to changes in cardiovascular function.

<div align="center">Gastric Emptying and Gut Motility</div>

In the presence of a GLP-1 infusion, the primary GLP-1 effect after a meal appears to be a reduced rate of gastric emptying in both healthy subjects and those with type 2 diabetes (206-209). This is demonstrated with significantly reduced postprandial glucose levels with GLP-1 infusion, when compared to saline infusion, and appropriately elevated insulin levels with the increased glycemia (208; 210). Physiological mechanisms that contribute to this effect include: decreased gastric acid secretion, decreased antral and duodenal contractions, as well as increased pyloric tone (211; 212). In addition, it has been shown that GLP-1 slows nutrient transit through the gut (213). It appears as though the effect of GLP-1 on gastric emptying and gut motility is important for physiological regulation of nutrient utilization, because endogenous plasma GLP-1 levels have a strong negative correlation with gastric emptying after a meal (214).

Slowed gastric emptying induced by elevated GLP-1 levels increases the sensation of fullness, along with decreasing the desire to eat (215). This is most likely attributable to increased stomach distention (216); however, this may not be the direct result of increased stomach content. Injection of GLP-1 and its analogues are known to induce nausea, especially during the first several treatments; therefore, decreased food intake may actually be due to nausea associated with acute treatment of GLP-1 and its mimetics.

Chronic Effects on Weight

Chronic GLP-1 administration in obese humans results in a significant decrease in body weight (217). Decreases in body weight occur with decreased energy intake, increased energy expenditure, or both. GLP-1 injection or infusion directly results in decreased food intake (218). As mentioned earlier, GLP-1 slows gastric emptying, which results in an increased sense of fullness, which could contribute to a decrease in food intake (215). In addition, those chronically treated with GLP-1 or its mimetics often experience nausea, decreasing the desire to eat, thus resulting in decreased food intake. Although these factors may contribute, it has been shown that intracerebroventricular, but not peripheral, administration of GLP-1 results in decreased food and water intake in rats, suggesting a centrally mediated effect (219; 220). This effect has been shown to be a direct effect on the regulation of energy homeostasis, and separate from the centrally mediated effect of gastric emptying and decreased satiety due to distention (221).

As previously mentioned, GLP-1R have been found throughout the brain (91), with the greatest density in the hypothalamus (222), specifically within the paraventricular and arcuate nucleus (219; 223). The hypothalamus, and in particular the arcuate nucleus, is a key site in the regulation of energy balance (224). GLP-1 stimulates proopiomelanocortin (POMC) neurons in a dose dependent manner (225). POMC neurons are located in the arcuate nucleus, and stimulation results in a significant decrease in food intake and body weight (226; 227). In addition, within the hypothalamus, GLP-1R are found co-localized with both GLUT2 and glucokinase, two proteins which serve the brain as glucose sensors (219; 228). This indicates that GLP-1

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activity in the hypothalamus is responsible for the decrease in food intake, and possibly takes part in glucose sensing in the brain.

The other side of the energy balance equation is energy expenditure. There is some evidence that GLP-1 also alters energy expenditure. GLP-1, infused either peripherally or i.c.v., increases basal metabolic rate via a mechanism initiated in the hindbrain, associated with increased heart rate and temperature (229). This is in concordance with increased basal metabolism in mice with elevated circulating GLP-1 levels due to ablation of DPP-IV (230). It has been shown that i.c.v. administration of GLP-1 does not alter rat locomotor activity (220).

Weight loss also been associated with chronic treatment of subcutaneous injections of exenatide, despite the peripheral route of delivery (231); however, this drug is administered at much higher and longer lasting levels than endogenous GLP-1 levels or even pharmacological levels of the peptide. This may result in exenatide actions that are not physiologically relevant; however, using what is known about GLP-1 and chronic weight loss, there could be several explanations for the weight loss. First, this may be the result of decreased food intake due to increased fullness as a result of gastric emptying, or nausea induced by treatment. Second, exenatide may be crossing the blood brain barrier to stimulate the POMC neurons. Third, exenatide may be initiating a central effect by interaction at the area postrema of the brain, which is known to increase neural activation with peripheral GLP-1 infusion (232). Fourth, it may be increasing basal energy expenditure, which has also been shown to be the result of peripheral GLP-1 administration (229).

The effect of GLP-1 on weight loss has been determined with the use of pharmacological doses of the peptide. At this point it is unclear if GLP-1 that is either endogenously produced in the CNS (233) or that which is released from the L cells in the gut contribute to the physiological regulation of energy balance.

Glucose Utilization Mediated by Intraportal Sensors

When GLP-1 is secreted from the L cell, it enters the circulation that empties into the portal vein. Due to its proximity to the site of secretion, and the rapid degradation of GLP-1, postprandial hepatic portal vein GLP-1 levels are significantly greater than those in the periphery (47). Immunohistochemistry has been used to show that GLP-1R are expressed on vagal afferent neurons that innervate the hepatoportal region (87). Intraportal injection of a physiological concentration of GLP-1 significantly increased afferent discharges from the hepatic branch of the vagus nerve in anesthetized rats (96; 234). This effect was not observed with intraportal injection of GIP (234). These studies indicate that the hepatic portal vein is a potential site of initiation of some of the indirect effects of GLP-1.

As mentioned earlier, the increase in afferent discharges from the hepatic branch of the vagus nerves is accompanied by increases in the efferent impulses in the pancreatic branch of the vagus nerves, an effect which is lost with hepatic vagotomy (96). This indicates that the neural signals, initiated at the hepatic portal vein, could increase signaling the pancreas and thereby participate in the incretin effect. Studies done in rats in the presence of an intraportal glucose infusion showed that GLP-1 delivered either intraportally or peripherally increases insulin secretion; however, this effect was

abolished in those that received GLP-1 intraportally with peripheral administration of a pharmaceutical ganglionic blocker (235). This ablation was not observed with the peripheral administration of GLP-1 (235). This suggests that the incretin effect may be the combination of two effects: 1) a neural reflex, initiated by intraportally delivered GLP-1, 2) direct interaction of GLP-1 at the β-cell.

Others have suggested that intraportal GLP-1 delivery initiates other effects of the peptide. GLP-1$^{-/-}$ mice are unable to increase whole body glucose uptake as a result of intraportal glucose infusion in contrast to control animals (236). It has also been shown that intraportal GLP-1 decreases glucose levels, without changing insulin levels in dogs (49; 237). Our lab has shown that when GLP-1 was infused peripherally at a superphysiological GLP-1 level, the peptide increased glucose utilization (48; 177). The super-physiological levels of GLP-1 may have overridden the sensing of an arterial-portal venous GLP-1 gradient or have allowed sufficient GLP-1 to contact the hepato-portal GLP-1 receptors. We have also infused a physiological level of GLP-1 into either a peripheral vein, the hepatic portal vein, or the hepatic artery, with similar increased hepatic glucose uptake in the latter two groups (47). However, these studies were not done in the presence of intraportal glucose delivery (47; 48; 177). It has been suggested that intraportal glucose delivery is necessary to observe the effect of intraportal GLP-1 on whole body glucose utilization (49; 236). Therefore, the physiological importance of intraportal GLP-1 delivery in glucose homeostasis has yet to be fully determined.

Specific Aims

Endogenous secretion of GLP-1 results in physiological elevations of the peptide at varying concentrations throughout the body. After a meal GLP-1 levels in the blood surrounding the L cell are double those in the hepatic portal vein (58), and levels in the hepatic portal vein twice those in the periphery (47). The overlying hypothesis of this thesis is that these specific elevations in GLP-1 level have a role in glucose regulation.

Specific Aim I was to determine if the effects of GLP-1 in the dog are mediated by delivery of the peptide into the hepatic portal vein. Changes in neural firing that occur upon GLP-1 entrance in the hepatic portal vein (96) may initiate portions of the incretin effect, or other glucoregulatory effects that are summarized in Figure 1.4. We evaluated changes in carbohydrate metabolism, due to both direct and indirect effects of GLP-1, *in vivo*. This was done in healthy dogs under conditions of hyperglycemia, with no pancreatic clamp, so hormones could freely respond. Intraportal glucose delivery was administered to mimic postprandial hepatic portal vein glucose levels and peripheral glycemia was maintained by peripheral infusion. We evaluated animals that experienced an elevation in GLP-1 levels that mimicked a postprandial state, induced by an intraportal GLP-1 infusion. This included a two-fold greater GLP-1 level in the hepatic portal vein than in the periphery. Comparisons were then made to animals that experienced an elevation of GLP-1 to match levels observed at the liver and periphery due to administration of the peptide via the hepatic artery. The glucoregulatory response in
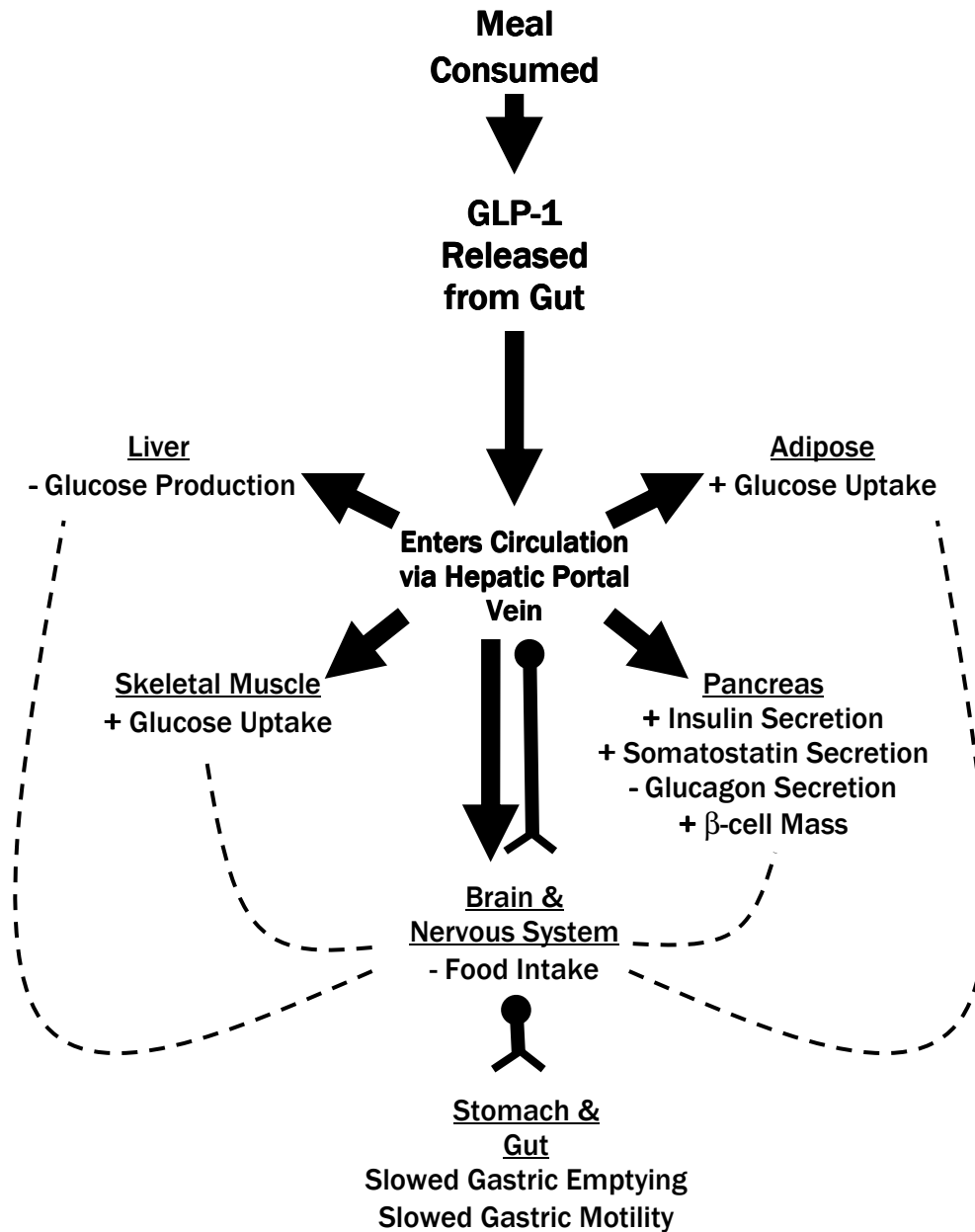
Figure 1.4 The effects of GLP-1 on glucose homeostasis. Solid arrows represent known direct effects and solid neurons represent known neural signaling. Dashed lines represent possible indirect effects resulting from neural signaling.

animals that had elevated GLP-1 levels, due to either intraportal or peripheral infusion, was then compared to animals that only experienced a matched hyperglycemia.

It is well understood that the majority of the acute effects of GLP-1 are glucose dependent. Specific Aim II was designed to determine if the route of glucose delivery, by which glycemia is increased, alters GLP-1 action. GLP-1 is only endogenously released after meals, with no secretion when hyperglycemia is achieved via infusion into a peripheral vein; therefore, under physiological conditions, GLP-1 secretion is always accompanied by glucose elevation in the hepatic portal vein. To determine if a glucose gradient between the hepatic portal vein and arterial blood must exist for GLP-1 to exert the effects that were observed in Specific Aim I, we replicated the conditions of the earlier aim with the exception of the intraportal glucose infusion. Hyperglycemia was maintained only by peripheral glucose infusion alone, and dogs received either intraportal GLP-1 or saline. Once again, this intraportal GLP-1 infusion resulted in levels that mimicked those observed postprandially.

GLP-1 possibly exerts its effects on whole body glucose utilization by increasing insulin sensitivity. It had yet to be determined how differences in β-cell glucose sensitivity or whole body insulin sensitivity could affect the actions of a physiological increase in GLP-1 levels. Specific Aim III was to determine if the state of whole body insulin responsiveness or β-cell glucose sensitivity alters the effectiveness of portally delivered GLP-1. To address this issue, we compared the results of Specific Aim I to results obtained in healthy animals that had been fasted for a shorter duration than those used earlier. It is well documented that duration of fast is negatively correlated with insulin sensitivity; therefore, in this aim we used a model of greater insulin sensitivity to

evaluate the effects of GLP-1. Glycemic levels were elevated by a combination of intraportal and peripheral glucose infusions and accompanied by an intraportal infusion of either GLP-1 or saline.

Specific Aim IV was to determine if endogenously released GLP-1 has any impact on postprandial glucose disposal in the dog. Although intraportal GLP-1 infusion does mimic the entrance of GLP-1 into the circulation, it does not replicate its secretion from the gut where it may enter the lymph or interact with nerve endings close to the site of secretion. As previously mentioned, upon release by the L cell, GLP-1 enters both the intestinal lymph system and portal circulation, with total levels of GLP-1 being approximately 5-6 times greater in the former than the latter (52). This presents the possibility that GLP-1 may be initiating indirect effects upstream of the portal circulation via neural activation. To execute this aim we studied healthy dogs and an additional subset of insulin resistant dogs, twice each. The animals received either a peripheral saline or exendin (9-39) infusion in random order. During both studies they received an orally delivered meal consisting of carbohydrate, fat, and protein. This allowed for the observation of any GLP-1 effects that may be mediated throughout the body, including close to the site of secretion.

CHAPTER II

MATERIALS AND METHODS

Animal Care and Surgical Procedures

Studies were carried out in conscious mongrel dogs of either sex. Sixty-three animals (18-27 kg) were maintained on a diet of meat (Kal Kan, Vernon, CA) and chow (Purina Lab Canine Diet No. 5006; Purina Mills, St. Louis, MO) composed of 34% protein, 14.5% fat, 46% carbohydrate, and 5.5% fiber based on dry weight. Three additional animals (29-35 kg) were maintained on a high-fat/high-fructose chow diet composed of 22% protein, 52% fat, 26% carbohydrate (over half of which is fructose). Water was available for all animals *ad libitum*. The animals were housed in a facility which met American Association for Accreditation of Laboratory Animal Care guidelines, and the protocol was approved by the Vanderbilt University Medical Center Animal Care Committee.

Approximately 16 days before experimentation, surgery was performed on the sixty-three animals (that received normal meat and chow) while under general anesthesia. Anesthesia was induced with propofol (given until induction) preceded by buprenorphine HCl (0.02 mg/kg, presurgery) 30 min earlier. Anesthesia was maintained by isoflurane (1.5-2.0% with oxygen) inhalation. The dog was placed in a supine position on a surgical table with an 8.5 mm inner diameter (ID) endotracheal tube (Concord/Protex, Kenee, NH), and ventilated with a tidal volume of 400 ml at 14 breaths per minute.

A laparotomy was performed by making a midline incision 1.5 cm caudal to the xyphoid process through the skin, subcutaneous layers and linea alba, and extending caudally 15-20 cm. For intraportal infusion of GLP-1, saline, and glucose (Specific Aims I, II, III), silastic infusion catheters (0.03 in ID; HelixMedical, Carpintera, CA) were placed in the following manner: A portion of the jejunum was exposed and a branch of a jejunal vein was selected for cannulation. A small section of the vessel was exposed by blunt dissection and ligated with 4-0 silk (Ethicon, Inc, Sommerville, NJ). A silastic infusion catheter was inserted into the vessel through a small incision and passed antegrade until the tip of the catheter lay approximately 1 cm proximal to the coalescence of two jejunal veins. Another silastic catheter was inserted into a distal branch of the splenic vein and advanced until the tip of the catheter lay 1 cm beyond the bifurcation of the main splenic vein. Catheters were secured in place with 4-0 silk. For GLP-1 infusion into the hepatic artery (Specific Aim I), a silastic infusion catheter (0.03 in ID; HelixMedical, Carpintera, CA) was inserted antegrade 3-4 cm into a hole created by an 18 gauge needle in the common hepatic artery. The catheter was secured with a purse string suture with using 5-0 polyester.

For blood sampling, silastic catheters (0.04 in ID) were placed into the left hepatic vein, the hepatic portal vein, left femoral artery, and the common iliac vein. The central and left lateral lobes of the liver were retracted cephalically and caudally, respectively. The left common hepatic vein and the left branch of the portal vein were exposed. A 14-gauge angiocath (Benton Dickinson Vascular Access, Sandy, UT) was inserted in the left branch of the portal vein 2 cm from the central liver lobe. A silastic catheter (0.04 in ID) for blood sampling was inserted into the hole created by the angiocath, advanced

retrograde about 4 cm into the portal vein so that the tip of the catheter lay 1 cm beyond the bifurcation of the main portal vein, and secured with three ties of 4-0 silk through the adventitia of the vessel and around the catheter. An angiocath was inserted into the left common hepatic vein 2 cm from its exit from the left lateral lobe. A silastic sampling catheter was inserted into the hole and passed antegrade 2 cm and secured into place with three ties of 4-0 silk suture.

Arterial blood was sampled from the left femoral artery, while venous blood for hindlimb balance in (Specific Aim IV) was sampled from the right iliac vein of three animals. The arterial sampling catheter was inserted into the left femoral artery following a cut-down in the left inguinal region. A 2 cm incision was made parallel to the vessel. The femoral artery was isolated and ligated distally. A silastic sampling catheter (0.04 in ID) was inserted and advanced 16 cm in order to place the tip of the catheter in the abdominal aorta. Exposure of the right proximal caudal femoral vein was achieved by blunt dissection, and the vessel was ligated distally. A silastic sampling catheter (0.03 in ID) was inserted and advanced into the common iliac vein and secured to the proximal caudal vein with 4-0 silk suture.

All catheters were filled with saline (Baxter Healthcare Corp, Deerfield, IL) containing 200 U/ml heparin (Abbott Laboratories, North Chicago, IL) and knotted. Abdominal catheters were secured to the abdominal wall and placed in a subcutaneous pocket prior to closure of the skin. The arterial and iliac venous sampling catheters were also placed in a subcutaneous pocket prior to closure of the skin.

Ultrasonic flow probes (Transonic System Inc, Ithaca, NY) were positioned around the hepatic artery and portal vein, to determine liver blood flow, and the external

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iliac artery, to determine hindlimb blood flow. The duodenum was laterally retracted to expose a section of the hepatic artery and portal vein. A small section of the portal vein was exposed by blunt dissection taking care not to disturb the nerve bundle located on the vessel. A 6 or 8 mm ID ultrasonic flow probe (Transonic Systems Inc, Ithaca, NY) was placed around the vessel. A small portion of the common hepatic artery was also carefully exposed and a 3 mm ID ultrasonic flow probe was secured around the vessel. The gastroduodenal vein was isolated and ligated to prevent blood from entering the portal vein beyond the site of the flow probe. Blood that would normally flow through the gastroduodenal vein was shunted through the caudal pancreatoduodenal vein draining the tail of the pancreas. A 4 mm ID flow probe was positioned on the external iliac artery. The ultrasonic flow probe leads were positioned in the abdominal cavity and secured with the ends of the catheters to the abdominal wall.

Two of the three animals that had been maintained on a high-fat/high-fructose diet received a partial pancreatectomy under general anesthesia, as described above. Following a midline incision and exposure to the abdominal viscera, the duodenum was retracted upwards and the pancreatico-duodenal vein and artery was isolated. The right lobe of the pancreas was isolated from the mesentery and transected using a surgical stapling device at the union of the right lobe caudal extremity and the distal duodenum. The previously isolated pancreatico-duodenal artery and vein were ligated and transected and the right lobe removed. Attention was directed to the left (splenic) lobe. The pancreatico-splenic veins and arteries were isolated and the left lobe was transected at the union with the pylorus. The mesenteric connections were transected. The arteries and veins were then cut and the left lobe removed. This resulted in removal of approximately

two-thirds of total pancreatic tissue. Hemostasis was controlled by electrocautery and/or vessel ligation with 4-0 silk suture.

After all abdominal surgeries, the subcutaneous layer was closed with a continuous suture of 2-0 chromic gut (Ethicon, Inc.). The skin was closed with horizontal mattress sutures of 3-0 Dermalon (Ethicon, Inc.). Immediately following surgery, the dogs received an intramuscular injection of penicillin G (10^6 U, Procaine; Anthony Products, Irwindale, CA) to minimize the possibility of infection. In addition, Flunixin (Meglumine 50mg/ml; Phoenix Scientific, Inc., St. Joseph, MO) was injected intramuscularly (1 mg/kg body weight) after wound closure for acute pain relief. Animals awoke from surgery within 2 h, were active, and ate normally approximately 8 h after surgery. Post-operatively, each dog also received 500 mg ampicillin (Principen; Bristol-Myers Squibb, Princeton, NJ) orally twice a day for 3 days.

All dogs studied had: 1) leukocyte count <18,000/mm^3, 2) a hematocrit >35%, 3) a good appetite, and 4) normal stools at the time of study. On the day of the experiment, the free ends of the catheters and ultrasonic leads were removed from their subcutaneous pockets under local anesthesia (2% lidocaine; Abbott Laboratories, North Chicago, IL). The contents of each catheter were aspirated, and they were flushed with saline. Blunt needles (18 gauge; Monoject, St. Louis, MO) were inserted into the catheter ends and stopcocks (Medex, Inc, Hilliard, OH) were attached to prevent the backflow of blood between sampling times.

Twenty gauge Angiocaths (Beckton Dickson) were inserted percutaneously into the left and right cephalic veins and into a saphenous vein for the infusion of tracers (Specific Aim I), dye (Specific Aim I, II, III), glucose (Specific Aims I, II, III) or exendin

9-39 (Specific Aim IV). Blood samples from animals maintained on a high-fat/high-fructose diet were conducted by deep venous sampling catheters placed in cephalic and/or saphenous veins. A continuous infusion of heparinized saline was started via the femoral artery, iliac vein, and deep venous sampling catheters. Animals were allowed to rest quietly in a Pavlov harness for at least 40 min before the start of the experiment.

In normal animals that were to be re-studied (Specific Aim IV): at the end of the experiment, anesthesia was induced with propofol and maintained with isoflurane (1.5-2.0% with oxygen), as described above. Catheters and ultrasonic flow probe leads, along with incisions, were washed and aseptically cleansed (3 times) with betadine/alcohol solution. Catheters were filled with a sterile mixture of heparin and glycerin (1000 U/ml in a 1:1 ratio). Their free ends were knotted, and along with the probe leads, were replaced in the subcutaneous pocket. Wound closure was performed as described above.

Experimental Design

Specific Aim I

Specific Aim I was to determine what actions of GLP-1 in the dog are mediated by delivery of the peptide into the hepatic portal vein.

Dogs were fasted for 42 h. This length of fast produces a metabolic state more closely resembling that in the overnight-fasted human (the dog takes much longer to absorb a meal) and results in liver glycogen levels that are at a stable minimum in the dog (238). The protocol consisted of a 100-min equilibration period (-140 to -40 min), 40-min basal sampling period (-40 to 0 min) and a 240-min experimental period (0 to 240 min). At t= -140, a continuous infusion of indocyanine green dye (0.076 mg/min; Sigma

Chemical, St. Louis, MO) used as a back-up method for hepatic blood flow measurement, along with a primed (30 μCi) continuous infusion of [3-^{3}H] glucose was started. At t=0, an intraportal glucose infusion (4 mg/kg/min) was started to activate the portal signal (239), in addition a peripheral glucose infusion (variable rate) was started to maintain the arterial plasma glucose level at 160 mg/dl. Also at t=0, an intraportal saline infusion was started in the CON group (n=8), an intraportal GLP-1 (7-36) infusion (1 pmol/kg/min; Bachem Biosciences, King of Prussia, PA) was started in the POR group (n=11) and a hepatic artery infusion of GLP-1 (7-36) (1 pmol/kg/min; Bachem Biosciences) was started in the HAT group (n=8). Pancreatic hormones were not clamped. This GLP-1 infusion rate creates plasma GLP-1 levels observed in the portal vein and arterial plasma after an oral glucose tolerance test (OGTT) in the dog (47). They are similar to a human's GLP-1 levels in response to an OGTT (43).

Specific Aim II

Specific Aim II was designed to determine if the route of glucose delivery alters GLP-1 action during hypgerglycemia.

Dogs were fasted for 42 h. The protocol consisted of a 100-min equilibration period (-140 to -40 min), a 40-min basal sampling period (-40 to 0 min) and a 240-min experimental period (0 to 240 min). At t= -140 min, a continuous infusion of indocyanine green dye (0.076 mg/min) was started. At t=0, a peripheral glucose infusion through a leg vein was started to achieve and maintain an arterial plasma glucose clamp at 160 mg/dl during in the experimental period (0 to 240 min). Also starting at t=0, animals received an intraportal infusion of either saline (SAL, n=6) or GLP-1(1

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pmol/kg/min; GLP-1, n=6). Pancreatic hormones were not clamped. Analysis of this group includes comparison to the intraportal saline and GLP-1 infusion groups from Aim I, in which an intraportal glucose infusion was present.

Specific Aim III

Specific Aim III was to determine if the state of whole body insulin responsiveness or β-cell glucose sensitivity alters the effectiveness of portally delivered GLP-1.

Dogs were fasted for 18 h, a shorter duration than in the previous protocols in order to improve whole body insulin responsiveness and β-cell glucose sensitivity. The protocol consisted of a 100-min equilibration period (-140 to -40 min), and 40-min basal sampling period (-40 to 0 min) and a 240-min experimental period (0 to 240 min). At t= -140, a continuous infusion of indocyanine green dye (0.076 mg/min) was started. At t=0, an intraportal glucose infusion (4 mg/kg/min), in addition to a peripheral glucose infusion (variable rate) to maintain a peripheral arterial plasma glucose clamp at 160 mg/dl, were started. Also starting at t=0, animals received an intraportal infusion of either saline (SAL, n=8) or GLP-1(1 pmol/kg/min; GLP-1, n=8). Pancreatic hormones were not clamped. Analysis of this group includes comparison to the intraportal saline and GLP-1 infusion groups from Aim I, which were fasted for 42 h.

<u>Specific Aim IV</u>

Specific Aim IV was to determine if endogenously released GLP-1 has any impact on postprandial glucose disposal in the dog.

Normal dogs (n=8) and insulin resistant dogs (n=3) were each studied twice, with a minimum of a one week interval separating experiments. After an 18-hour fast, the dogs underwent a 40-min acclimation period (-60 to -20 min), a 20 minute basal sampling period (-20 to 0 min), followed by a 320-min experimental period (0 to 320 min). At t=0, a peripheral infusion of saline or the GLP-1R antagonist exenatide (9-39) (500 pmol/kg/min) was started. Treatment and control studies were conducted in a random order. At t=30, all dogs received an orally administered liquid mixed meal consisting of 480 calories [63% carbohydrate (glucose polymer), 17% protein (whey), 20% fat (microlipid)], spiked with acetaminophen (500 mg), to quantify gastric empting.

Collection and Processing of Samples

Blood samples were drawn from the femoral artery and portal, hepatic, and iliac veins in normal dogs, and deep venous samples were drawn from the insulin resistant dogs (Specific Aim IV), at the predetermined time points. Additionally, whenever the experimental design required a glucose clamp (Specific Aims I, II, III), small (~0.5 ml) arterial samples were drawn every 5 min to facilitate maintenance of the plasma glucose concentration. Before samples were taken, the sampling catheter was cleared by withdrawing 5 ml of blood into a syringe. After sampling, this blood was re-infused and the catheter was flushed with heparinized saline (1 U/ml; Abbott Laboratories, North Chicago, Il). The total volume of blood withdrawn did not exceed 20% of the animal's

blood volume, and two volumes of normal saline (0.9% sodium chloride; Baxter Healthcare Co., Deerfield, Il) were given for each volume of blood withdrawn. No significant decrease in hematocrit occurred throughout duration of study.

Before the experiment started, an arterial blood sample was drawn and centrifuged (3000 rpm for 7 min). The plasma from this blood sample was used to prepare a 3% plasma/saline solution in which the GLP-1 (Specific Aims I, II, III) or exendin (9-39) (Specific Aim IV) were diluted. In addition, this plasma was used for the indocyanine green standard curve. When samples were taken from all vessels, the arterial and portal blood samples were collected simultaneously ~30 s before the collection of the hepatic and iliac venous samples in an attempt to compensate for the transit time through the liver and muscle, and thus allow for the most accurate estimates of net substrate balance.

After the final sampling point, anesthesia was induced with propofol (given until induction) in animals that awaited a repeat study (Specific Aim IV), and catheters were re-tucked as described above. Otherwise, normal animals were euthanized with pentobarbital (125 mg/kg). Insulin resistant animals had the deep venous sampling catheter removed, and were returned to the housing facility.

Immediately following each sample collection, the blood was processed. A 20 μl aliquot of arterial whole blood was used for the immediate duplicate measurement of hematocrit using capillary tubes (0.4 mm ID; Drummond Scientific Co., Broomall, PA). The remaining blood was placed into tubes containing potassium ethylenediaminetetraacetate (EDTA, 1.6 mg/ml; Sarsdedt, Newton, NC), inverted and gently mixed. One ml of whole blood was removed from the above tube and lysed with 3

ml of 4% perchloric acid (PCA; Fisher Scientific, Fair Lawn, New Jersey). The solution was vortexed, centrifuged, and the supernatant was stored for later determination of whole-blood metabolites (alanine, β-hydroxybutyrate, glycerol, and lactate). Another 1 ml aliquot was taken placed in a tube with 10 µl of DPP-IV inhibitor (Linco Research, St. Charles, MO) to ensure the integrity of the GLP-1. This aliquot was then centrifuged, and the supernatant was stored on dry ice for later determination of plasma GLP-1 levels. The remainder of the blood was centrifuged at 3000 rpm at 4º C to obtain plasma.

Four 10 µl aliquots of plasma were immediately analyzed for glucose using the glucose oxidase method with a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). A 1 ml aliquot of plasma received 50 µl of 10,000 KIU/ml Trasylol (FBA Pharmaceuticals, New York, NY) and was stored for analysis of glucagon and C-peptide. A 500 µl aliquot of plasma received 10 µl of tetrahydrolipostatin (3g/L) and was stored for analysis of non-esterified free fatty acids (FFA). The remainder of the plasma was used for analysis of $[3\text{-}^3H]$ glucose (Specific Aim I), insulin, indocyanine green (Specific Aims I, II, III), and acetaminophen (Specific Aim IV). After each sample was processed, it remained on wet ice for the remainder of the experiment and was then stored at -70º C until analysis was performed.

Sample Analysis

Plasma Glucose

Plasma glucose concentrations were determined by the glucose oxidase method (240) using a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). The reaction sequence was as follows:

$$\text{ß-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid and } H_2O_2 \tag{1}$$

$$H_2O_2 + \text{ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde} + H_2O \tag{2}$$

$$H_2O_2 + 2H^+ + 2I^- \xrightarrow{\text{molybdate}} I_2 + H_2O \tag{3}$$
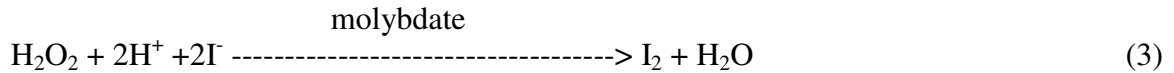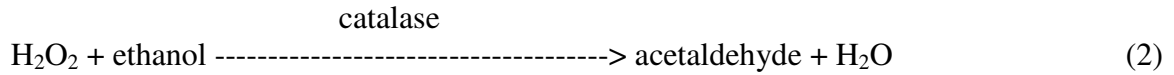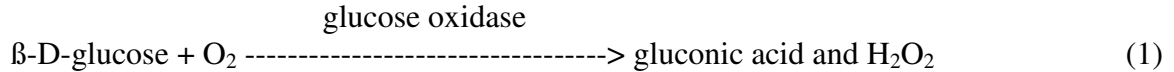
The glucose concentration is proportional to the rate of oxygen consumption. The plasma glucose concentration in a sample (10 μl) is determined by comparison of the oxygen consumption in the samples with the rate of oxygen consumption by a standard solution (150 mg/dl). There is no end-product inhibition of the process, as reactions 2 & 3 remove all of the hydrogen peroxide. Thus virtually all of the glucose in the sample is consumed. Plasma glucose was measured 4 times at each sampling time point for each vessel and a minimum of 2 times for samples drawn to clamp glucose. The glucose analyzer is accurate to 450 mg/dl. In glucose balance calculations, plasma glucose levels were converted to whole blood values using a previously determined correction factor, which assumes blood glucose to be 73% of the plasma glucose values (241).

Plasma [3-^{3}H] glucose

For assessment of plasma [3-^{3}H] glucose (Specific Aim I), samples were deproteinized according to the method of Somogyi-Nelson (242-244). Immediately following each experiment 1 ml aliquots of plasma were mixed with 5 ml of 0.067 N Ba(OH)$_2$ and 5 ml of 0.067 N ZnSO$_2$ (Sigma Chemical). For 1-3 days, these samples were kept at 4°C, after which they were centrifuged at 3000 rpm for 20 min. A 5 ml aliquot of the supernatant was pipetted into a glass scintillation vial and placed in a heated vacuum oven to evaporate ^{3}H$_2$O. The residue was reconstituted in 1 ml of

deionized water and 10 ml liquid scintillation fluid (EcoLite (+); Research Product

Division, Costa Mesa, CA), and placed in Beckman LS 9000 Liquid Scintillation Counter

(Beckman Instruments Inc, Irvine, CA) for counting. The scintillation counter was

programmed so that the processor corrected the counts per minute (cpm) for quenching of

the radioactivity in the sample and presented the results as disintegrations per minute

(dpm).

To assess the loss of radioactive glucose during the deproteinization process, a

recovery standard was prepared. The [3-^{3}H]glucose infusate was diluted 1:250 (vol:vol)

with saturated benzoic acid containing 1 mg/ml cold glucose. Six 1 ml aliquots of this

diluted ^{3}H infusate were placed into 2 sets of glass scintillation vials labeled as chemical

standard evaporated (CSE) or chemical standard (CS); therefore CSE and CS were

measured in triplicate. The diluted infusate aliquots in the CSE vials were evaporated to

dryness (with plasma samples) in a heated vacuum oven and reconstituted with 1 ml

deionized water. The diluted infusate aliquots in the CS were not evaporated.

Scintillation fluid (10 ml) was added to all standard vials and the standards were counted.

Three additional 1 ml aliquots of diluted ^{3}H infusate were treated identical to the plasma

samples and labeled chemical recovery standard (CRS). Comparison of the CS and CSE

provided an evaluation of the loss of ^{3}H counts in the evaporation process. The final

amount of radioactivity per sample was determined by generating a recovery factor (ratio

of radioactivity in the CSE compared to CRS) which accounted for the radioactivity lost

during sample processing.

Whole blood concentrations of alanine, b-hydroxybutyrate, glycerol, and lactate were determined using the methods developed by Lloyd *et al*. (245) for the Technicon Autoanalyzer (Tarrytown, NY) and were modified for the Packard Multi Probe Robotic Liquid Handling System (Perkin Elmer; Shelton, CT). Enzymes and coenzymes for metabolic analyses were obtained from Boehringer-Mannheim Biochemicals (Germany) and Sigma Chemicals. The reduced form (NADH) has a native fluorescence, which is not exhibited in the oxidized form. Excess amounts of NAD and enzyme/coenzyme are added to the metabolite samples. NAD is reduced to NADH upon oxidation of the metabolite. A fluorometer incorporated in the system detects changes in fluorescence resulting from changes in NADH concentration; therefore, the concentration of the metabolite present is proportional to the NADH produced.

Metabolites were measured in the PCA-treated blood samples as described above. A standard curve was constructed for each metabolite using known concentrations of the analyte prepared in 3% PCA. The Packard Multi Probe Robotic Liquid Handling System pipettes the sample into one well of the 96-well plate. After an initial absorbance is read, the Packard Multi Probe Robotic Liquid Handling System pipettes enzyme solution into each well and shakes the plate to mix sample and enzyme. The reaction proceeds and after an allotted time, the change in absorbance is determined. All assay reactions are reversible, with the exception of glycerol kinase. The NAD and enzyme are in excess compared to the substrate, thus the reactions are essentially taken to completion and the rate-limiting component is the substrate; therefore, all reactions below are written with a single direction arrow. All reactions are carried out at 23°C.

The alanine assay involved the reaction:

alanine dehydrogenase

L-alanine + NAD$^+$ + H$_2$O ------------------------------> Pyruvate + NADH + NH$_4^+$ (4)
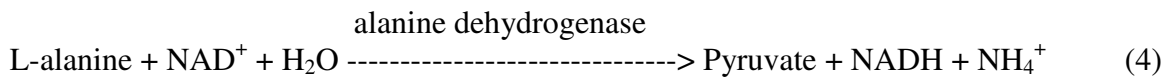
The enzyme buffer used was 0.05 M trizma base, 2 mM EDTA and 1 mM hydrazine

hydrate, pH 10. To 10 ml of enzyme buffer, 4.6 mg of NAD and 3.4 Units (U) of alanine

dehydrogenase were added.

<u>ß-hydroxybutyrate</u>

The ß-hydroxybutyrate analysis involved the following reaction:

3-hydroxybutyrate dehydrogenase

ß-hydroxybutyrate + NAD$^+$ -----------------------------------> acetoacteate + NADH + H$^+$ (5)
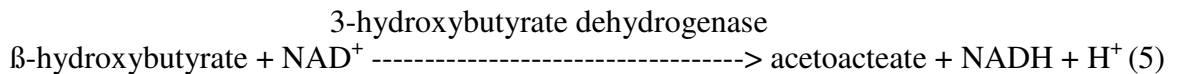
The enzyme buffer was 0.2 M monopotassium phosphate, 3 mM EDTA and 1 mM

hydrazine hydrate, pH 8.5. To 10 ml of enzyme buffer, 12 mg NAD and 2.1 U ß-

hydroxybutyrate dehydrogenase were added.

<u>Glycerol</u>

The glycerol assay involved the following reactions:

$$\text{glycerol + ATP} \xrightarrow{\text{glycerokinase}} \text{glycerol-l-phosphate + ADP} \qquad (6)$$

$$\text{L-glycerol-l-phosphate} + NAD^+ \xrightarrow{\text{glycerol-3-phosphate dehydrogenase}} \text{dihydroxyacetone phosphate} + NADH + H^+ \qquad (7)$$
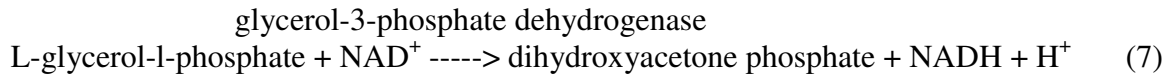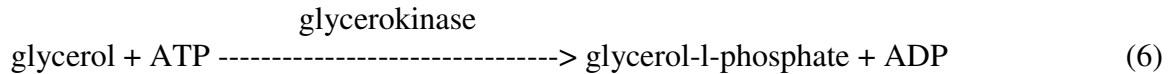
The enzyme buffer was 0.09 M glycine, 1 mM hydrazine, and 0.01 M $MgCl_2$, pH 9.5. To 10 ml of the enzyme buffer, 15.4 g NAD, 15.4 mg ATP, 0.3 U glycerokinase, and 0.6 U glycerol-3-phosphate dehydrogenase were added.

Lactate

The lactate assay involved the following reaction:

$$\text{lactate} + NAD^+ \xrightarrow{\text{lactate dehydrogenase}} \text{pyruvate} + NADH + H^+ \qquad (8)$$
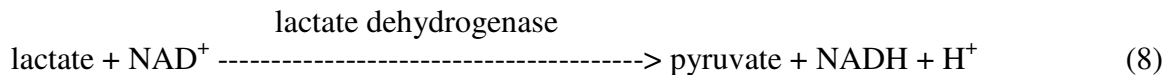
The enzyme buffer used was 0.24 M glycine and 0.25 M of hydrazine dihydrochloride and 7 mM disodium EDTA, pH 9.6. To 10 ml of enzyme buffer, 4.6 mg NAD and 0.1 U lactate dehydrogenase were added.

Plasma Free Fatty Acids (FFAs)

Plasma FFA levels were determined spectrophotometrically using the Packard Multi Probe Robotic Liquid Handling System and a kit obtained from Wako Chemicals (Richmond, VA). This assay was performed at 37°C. The following reactions were used in the analysis:

$$\text{FFA + ATP + CoA} \xrightarrow{\text{acyl-CoA synthetase}} \text{acyl-CoA + AMP + PPi} \qquad (9)$$

$$\text{acyl-CoA} + O_2 \xrightarrow{\text{acyl-CoA oxidase}} \text{2,3-trans-enoyl-CoA} + H_2O_2 \qquad (10)$$

$$2H_2O_2 + 3 \text{ methyl-N-ethyl-N-(\ss-hydroxyethyl)-aniline} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}}$$

purple color adduct (11)
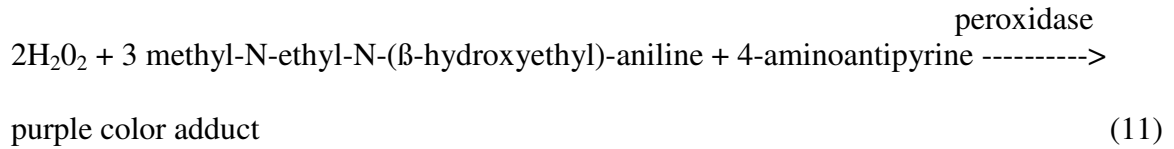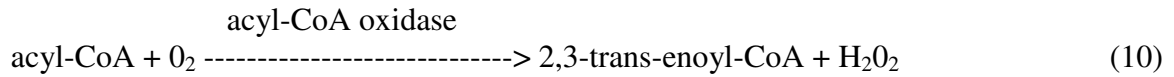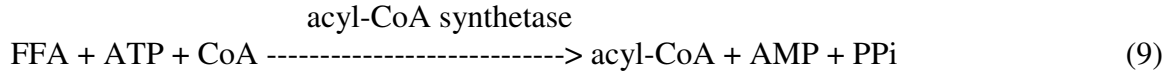
The purple colored adduct is measured at an optical density of 550 nm and is proportional

to the FFA concentration in the sample. The FFA values are then obtained from a

calibration curve with known amounts of oleic acid.

Plasma Hormones

The plasma hormone levels of insulin, glucagon, and C-peptide were measured

using radioimmunoassay (RIA) techniques (246). In general, a sample containing an

unknown amount of hormone was incubated with an antibody specific for that hormone.

A known amount of radiolabeled hormone was added to the mixture to compete with the

antibody binding sites. A double antibody procedure which caused precipitation of the

bound complex was used to separate unbound hormone from the antibody-hormone

complexes. The radioactivity of the precipitate was measured via a Cobra II Gamma

Counter (Packard Instrument Co., Meriden, CT). Binding of the radiolabeled hormone is

inversely proportional to the amount of unlabeled hormone present, and a standard curve

was constructed using known concentrations of unlabelled hormone.

Insulin

Immunoreactive plasma insulin was measured using a double-antibody RIA procedure (7). A 100 µl aliquot of the plasma sample, 200 µl of ^{125}I-labled insulin, and 100 ml of guinea pig specific antibody to insulin (both from Linco Research, Inc., St. Charles, MO.) were mixed and incubated for 18 h at 4°C. The sample was then treated with 100 µl goat anti-guinea pig IgG (2nd antibody) and 100 µl IgG carrier and incubated for 30 min at 4°C. One ml of a wash buffer was added and the tubes were centrifuged at 3000 rpm. The samples were decanted and the portion of total radioactivity bound to the antibody (pellet) was counted in a Cobra II Gamma Counter (Packard Instrument Co, Meriden, CT).

The log of the amount of hormone in the sample was inversely proportional to the log (bound label/free label). The insulin concentration in each sample was determined by comparison to a standard curve constructed using known amounts of unlabeled hormone. The samples were corrected for non-specific binding. The sample detection range was 1-150 µU/ml. The specificity of the antibody is 100% to porcine, canine, and human insulin, but also cross reacts with bovine insulin (90%), human proinsulin (38%), and the split proinsulin products Des 31,32 (47%) and Des 64,65 (72%). In general, less than 15% of the basal insulin level is due to non-insulin cross reactivity (mainly the split proinsulin products Des 31,32 and Des 64,65). There is no cross reactivity to glucagon, pancreatic polypeptide, C-peptide, or somatostatin. The recovery for the assay was between 90-100% based on spiking the sample with known amounts of insulin, and the interassay CV was approximately 7-8% for the entire range of the dose response curve.

Glucagon

Immunoreactive plasma glucagon was also measured using a double antibody

RIA (Linco Research, Inc., St. Charles, MO) (247). The protocol utilized primary and

secondary antibodies specific for glucagon (kit with glucagon antibodies and ^{125}I tracers

from Linco). A 100 µl aliquot of the plasma sample and 100 µl of guinea pig specific

antibody to glucagon were mixed and incubated for 24 hours at 4°C. Next, 100 µl of ^{125}I-

labeled glucagon was added and the solution was incubated for an additional 24 h at 4°C.

The samples were then treated with 100 µl goat anti-guinea pig IgG (2nd antibody) and

100 µl IgG carrier and incubated for 2 hours at 4°C. One ml of a wash buffer was added

and the tubes were centrifuged at 3000 rpm. The samples were decanted and the portion

of total radioactivity bound to the antibody (pellet) was counted in a Cobra II Gamma

Counter.

The log of the amount of hormone in the sample was inversely proportional to the

log (bound label/free label). The glucagon concentration in each sample was determined

by comparison to a standard curve constructed using known amounts of unlabeled

hormone. The samples were corrected for non-specific binding, and the sample detection

range was 20-400 pg/ml. The antibody is 100% specific to glucagon with only slight

(0.01 %) cross reactivity to oxyntomodulin, and no cross reactivity with human insulin,

human proinsulin, human C-peptide, glucagon-like petide-1, somatostatin, or pancreatic

polypeptide. A cross-reacting protein reads in this assay and results in a glucagon free

sample reading of 15-20 pg/ml above the actual glucagon level. This represents a stable,

constant background in all samples. The recovery for the assay was between 80-109%

based on spiking the sample with known amounts of glucagon, and the interassay CV was approximately 6-10% for the entire dose response curve.

<u>C-peptide</u>

Canine C-peptide was also measured using a double antibody disequilibrium procedure similar to that used for glucagon (248). Kits containing canine C-peptide antibody and ^{125}I tracer were obtained from Linco Research. A 100 µl aliquot of the plasma sample and 100 µl of guinea pig-specific antibody to canine C-peptide were mixed and incubated for 24 h at 4°C. Then 100 µl of ^{125}I-labled canine C-peptide was added and incubated for an additional 24 h at 4°C. The sample was then incubated with 1 ml of precipitating agent containing guinea pig IgG antibody and carrier for 30 min at 4°C. Tubes were centrifuged, decanted, and the portion of total radioactivity bound to the antibody (pellet) was counted in a Cobra II Gamma Counter.

The log of the amount of hormone in the sample was inversely proportional to the log (bound label/free label). The C-peptide concentration in each sample was determined by comparison to standards dissolved in plasma using known amounts of unlabeled hormone. Dog plasma was treated with charcoal (1% w:v) to remove immunoreactive contaminants. Samples were corrected for non-specific binding. The sample detection range was 0.1-10 ng/ml. The antibody is 100% specific for canine C-peptide with no cross-reactivity to rat C-peptide, human C-peptide, human proinsulin, bovine proinsulin, porcine proinsulin, or glucagon. The recovery of the assay was approximately 90%.

Glucagon-Like Peptide-1 (GLP-1)

Plasma GLP-1 levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Linco Research, Inc.). A 100 µl aliquot of plasma (pretreated as described in *Collection and Processing of Samples*) and 100 µl of assay buffer were manually pipetted into a well, of a 96-well plate, bound with monoclonal antibodies specific to the N-terminal region of active GLP-1 molecules, and incubated at 4°C, overnight. After a series of washes, 200 µl of anti-GLP-1 alkaline phosphatase detection conjugate was added to the well and incubated for 2 h at room temperature. After a second series of washes, 200 ml of methyl umbelliferyl phosphate (MUP) was added and incubated in the dark for 25 min at room temperature. MUP, in the presence of alkaline phosphatase, forms the fluorescent product umbelliferone. This reaction was stopped with 50 µl of stop buffer. Immediately after stop solution was added, excitation/emission wavelength was read at 365/450 nm on a fluorescence plate reader (Packard Fusion, PerkinElmer, Waltham, MA).

All samples are pipetted in duplicate, and read by the plate reader three times. The amount of fluorescence generated is directly proportional to the concentration of active GLP-1 in the sample. Quantification of the GLP-1 level in the plasma sample is derived from a standard curve run on the same plate as the samples. This assay is highly specific for active forms of GLP-1 [GLP-1 (7-36) amide and GLP-1 (7-37)], with no cross-reactivity to other forms of GLP-1 (e.g., 1-36 amide, 1-37, 9-36 amide, 9-37). This assay is reliable in a range of 2-100 pM.

<u>Acetaminophen</u>

Arterial plasma acetaminophen levels were determined using a modified protocol designed for high performance liquid chromatography (HPLC) (249). A 500 ml aliquot of plasma was spiked with 20 μl of 2-acetaminophenol (40 μg/ml) to serve as an internal standard (250). Equal volumes of spiked plasma, 0.3 N barium hydroxide, and 0.3 N zinc sulfate were mixed and incubated on ice for 5 min. The sample was then spun at 4°C at 3000 rpm for 10 min. The decanted supernatant was dried using vacuum centrifugation (Speedvac Concentrator, Savant SVC 200H, Thermo Scientific, Waltham, MA). The sample was then reconstituted with 200 μl of a 10% methanol/water solution (v:v).

A 50 μl aliquot was injected for delivery into the HPLC column (uBondapak C18 3.9X30 w/guard), at a temperature of 45°C. The mobile phase A (5% methanol/water, v:v) and B (15% methanol/water, v:v) were set at a combined flow rate of 0.4 ml/min for the entire duration of assay. With the profile curve indicating the type of transition from one setting to the next, the gradient for the mobile phase was set as follows: initial setting at 100% A, 0 profile curve; t=4 min at 100% A, 11 profile curve; t=20 min at 75% A, 25% B, 6 profile curve; t=30 min at 100% B, 7 profile curve; t=40 min at 100% A, 11 profile curve. Total run time of 64 min, with an 18 min acquisition delay. Fluorescence was measured with variable wavelength UV detector (Waters 481, Millipore, Billerica, MA) set at 240 nm at 0.5 AUFS.

Peak area as identified by the ESA 500 Chromatograph and data station are representative of acetaminophen concentration. Peak area increases in a linear fashion, proportional to acetaminophen concentration; therefore, sample concentration is determined as a ratio of acetaminophen peak area in the sample to internal standard peak

area. This assay has been confirmed to be accurate up to concentrations of 40 µg/ml. CV for assay was 4%.

Blood Flow

Blood flow in the hepatic artery, hepatic portal vein blood, and common iliac vein were determined using ultrasonic flow probes implanted during surgery (as described in *Animal Care and Surgical Procedures*). Total hepatic blood flow (the sum of blood flow in the hepatic artery and the hepatic portal vein) was also assessed using the indocyanine green (ICG) dye method (Specific Aims I, II, III), according to Leevy *et al*. (251). The results presented in this document were calculated using ultrasonic determined flow, as this method allows for the direct measurement of blood flow in the hepatic artery and hepatic portal vein, whereas the ICG dye method requires an assumption of the percent contribution of each vessel to total hepatic blood flow. ICG-determined flow was used as a backup measurement in the case of ultrasonic flow probe failure; however, the same conclusions were drawn regardless of method used to calculate the data.

Ultrasonic flow measurements represented instantaneous variations in velocity and, therefore, provided blood flow in individual vessels of interest. Each probe determined the mean transit time of an ultrasonic signal passed back and forth between two transducers within a probe which were located upstream and downstream of the direction of blood flow in the vessel. The transducers are made of piezoelectric material which is capable of both receiving and transmitting the ultrasonic signal. The downstream transducer first emits an ultrasonic pulse into the blood vessel that is received upstream by a second transducer. After the upstream transducer receives the

ultrasonic signal, it reemits the ultrasonic pulse signal back to the downstream transducer. The transit time of each ultrasonic beam, as measured by the upstream and downstream transducers (ΔT_{up} and ΔT_{down}, respectively), is defined by the following relationships:

$$\Delta T_{up} = D / (v_o - v_x) \tag{12}$$

$$\Delta T_{down} = D / (v_o + v_x) \tag{13}$$

where D is the distance traveled by the ultrasonic beam within the acoustic window of the probe, v_o is the phase velocity, or the speed of sound, in blood, and v_x is the component of fluid velocity that is parallel or antiparallel to the phase velocity. The parallel component augments the phase velocity when the signal is traveling in the same direction of blood flow, while the antiparallel component subtracts from phase velocity if the ultrasonic signal is moving against the flow of blood in the vessel. Combining the two expressions for transit time yields the following equation:

$$\Delta T_{up} - \Delta T_{down} = [D / (v_o - v_x)] - [D / (v_o + v_x)] \tag{14}$$

The transit times measured by both transducers, the distance traveled by the beam, and the speed of sound in blood are all known quantities; therefore, this equation can be used to calculate v_x. Once v_x is attained, the transit velocity (V) of blood traveling through the vessel can be determined according to the following equation:

$$V \cos \theta = v_x \tag{15}$$

where θ is the angle between the centerline of the vessel and the ultrasonic beam axis. Finally, blood flow is the product of the transit velocity and the cross-sectional area of the vessel. The cross-sectional area of the vessel is pre-determined by the size of the acoustic window according the probe model. Since transit time is sampled at all points across the diameter of the vessel, volume flow is independent of the flow velocity profile.

The ICG method is based on the Fick principle, according to which the net balance of a substrate across an organ equals the concentration difference of the substrate across the organ multiplied by the blood flow through the organ. The equation can be rearranged to calculate hepatic blood flow by dividing hepatic ICG balance by the arteriovenous difference of ICG across the liver. Because the liver is assumed to be the only site of ICG clearance, hepatic ICG uptake is equal to ICG infusion rate under steady state conditions. The extraction of ICG across the liver remains constant for brief infusions; however, if ICG is infused for a longer time (> 4 h), the dye level in plasma gradually increases, resulting in a 5-10 % overestimation of hepatic blood flow.

Arterial and corresponding hepatic vein plasma samples were centrifuged at 3000 rpm for 30 min, using no brake, to pellet the residue. Absorbance was then measured on a Spectronic spectrophotometer at 810 nm. This process was then repeated, and the values obtained for each sample were averaged. A standard curve was constructed by adding successive 5 µl aliquots of diluted dye (1:10 dilution) to 1 ml of plasma drawn from the animal before the dye infusion was started. The mean of the incremental changes in absorbance was then used to calculate hepatic plasma flow (HPF) as follows:

HPF = [IR x 10 x SCMD] / [dog weight in kg x (0.005) x (A-H)]   (16)

where IR is ICG infusion rate (ml/min), SCMD is the standard curve mean difference per 5 µl increments, and A-H is the difference in absorbance between the arterial and the hepatic venous sample. The value of 10 was used to correct for the dilution of the ICG used in the standard curve, and 0.005 was the volume in ml used as increments in the standard curve. Hepatic blood flow (HBF) was derived from HPF:

HBF=HPF/(1-hematocrit)   (17)

Hematocrit was measured at every time point of each in which samples were taken from the artery and portal and hepatic veins. This technique only determines total blood flow; therefore, an assumption was made regarding the contribution of blood flow in the vessels supplying the liver. The normal distribution of flow was assumed to be 20% artery and 80% hepatic portal vein at baseline, based on extensive historical data.

Calculations

Glucose Infusion Rate

The total glucose infusion rate (GIR) was determined by the following calculation:

$$GIR = GIR_{portal} + GIR_{peripheral}$$ (18)

where GIR_{portal} is the rate of glucose infused into the portal vein and $GIR_{peripheral}$ is the rate of glucose infused peripherally.

<u>Net Substrate Balance</u>

The net balance of a substrate across an organ, otherwise known as the arteriovenous (A-V) difference technique, utilized the Fick principle as described for the ICG-determination of blood flow (as described in *Sample Analysis* under *Blood Flow*) The net balance of a substrate (NSB) was calculated as:

$$NSB = Load_{out} - Load_{in} \tag{19}$$

or the difference between the substrate load leaving the region/organ ($Load_{out}$) and the substrate load reaching the region/organ ($Load_{in}$). This equation was applied to net hepatic substrate balance (NHSB), net splanchnic substrate balance (NSSB), net gut substrate balance (NGSB), and hindlimb substrate balance (HindSB).

For NHSB, the $Load_{in}$ was calculated according to the equation:

$$Load_{inhepatic} = ([S]_A \times HABF) + ([S]_{PV} \times PVBF) \tag{20}$$

where $[S]_A$ and $[S]_{PV}$ are arterial and portal venous substrate concentrations, respectively, and HABF, PVBF are hepatic artery and the portal vein blood flows, respectively.

For NHSB, $Load_{out}$ was calculated according to the equation:

$$\text{Load}_{\text{outhepatic}} = [S]_{HV} \text{ x HBF} \tag{21}$$

where $[S]_{HV}$ is the substrate concentration in the hepatic vein, and HBF is the total

hepatic blood flow.

For NSSB, the Load_{in} and Load_{out} were calculated as follows:

$$\text{Load}_{\text{insplanchnic}} = [S]_A \text{ x HBF} \tag{22}$$

$$\text{Load}_{\text{outsplanchnic}} = [S]_{HV} \text{ x HBF} \tag{23}$$

For NGSB, the Load_{in} and Load_{out} were calculated as follows:

$$\text{Load}_{\text{ingut}} = [S]_A \text{ x PVBF} \tag{24}$$

$$\text{Load}_{\text{outgut}} = [S]_{PV} \text{ x PVBF} \tag{25}$$

For HindSB, the Load_{in} and Load_{out} were calculated as follows:

$$\text{Load}_{\text{inhindlimb}} = [S]_A \text{ x IBF} \tag{26}$$

$$\text{Load}_{\text{outhindlimb}} = [S]_{FV} \text{ x IBF} \tag{27}$$

where IBF is iliac blood flow and $[S]_{FV}$ is the concentration of the substrate sampled from

the femoral vein. Blood flows were used for all substrate balance calculations except

GLP-1 across the liver, for which plasma flows were used.

Net Substrate Clearance

Net substrate clearance (NSC) across an organ/region is calculated as follows:

$$NSC = NSU / [S] \tag{28}$$

where NSU is net substrate uptake across an organ/region and [S] is the substrate

concentration entering that organ/region.

Net Hepatic Substrate Fractional Extraction

Net substrate fractional extraction across the liver (NHSFE) was calculated as:

$$NHSFE = NHSU / Load_{inhepatic} \tag{29}$$

where NHSU is the net hepatic substrate uptake.

Hepatic Sinusoidal Substrate Level

Hepatic sinusoidal substrate level (HSSL) was calculated as:

$$HSSL = ([S]_A \times HABF/HBF) + ([H]_{PV} \times PVBF/HBF) \tag{30}$$

where $[S]_A$ and $[S]_{PV}$ are arterial and portal venous substrate concentrations, respectively,

and HABF, PVBF and HBF are hepatic artery, portal vein and total hepatic blood flows,

respectively.

Glucose Turnover

Glucose turnover is the rate at which old glucose is replaced with new glucose. Glucose production (R_a) and glucose utilization (R_d) were determined using an isotope dilution method described by Wall (252), as simplified by DeBodo (253) and using a two-compartmental model (254) with canine parameters (255). The glucose pool was initially primed with an injection of [3-^{3}H]glucose followed by a constant infusion of the tracer. By the beginning of the control period, the tracer ([3-^{3}H]glucose) and tracee (cold glucose) were in equilibrium so that the specific activity of glucose (SA = dpm glucose/mg glucose) was in a steady state. R_a and R_d were calculated according to the following equations:

$$R_a = [I - N \, (dSA/dt)]/SA, \text{ and} \tag{31}$$

$$R_d = R_a - (dN/dt) \tag{32}$$

where I is infusion rate of tracer (dpm/min), N is the pool size of glucose (mg) and t is time (min) (256). In a steady state, when $dSA/dt = 0$, the R_a equation is simplified to:

$$R_a = I/SA \tag{33}$$

This method utilizes a one-compartment model of glucose kinetics as described by Steele (257). Assumptions of the model are that one compartment of glucose consists of both rapidly mixing and slowly mixing glucose pools; therefore, when a rapid change

in the cold glucose concentration is induced in the system, the consequent changes in

glucose specific activity would be unevenly distributed throughout the entire glucose

compartment. To compensate for this problem, the pool size is calculated as:

$$N = pVC \tag{34}$$

where p is the pool fraction, V is the volume of distribution of glucose (ml) and C is

concentration of cold glucose (mg/dl). The pool fraction (the rapidly mixing component

of the glucose compartment) was estimated to be 0.65, or 65 % of the total system (258),

while V was assumed to be the extracellular volume, which is approximately 22% of the

dog weight (259).

The major limitation of the one-compartment model is that a rapid change in SA

invalidates the method, so that a fall in SA, which occurs either by endogenous glucose

production or exogenous glucose infusion in the presence of a constant [3-^{3}H] glucose

infusion, the change in SA would cause an error in the estimation of R_a (underestimation

if SA drops, overestimation if SA increases) (260). Two approaches have been applied to

solve this problem. One strategy is to reduce the change in glucose SA by

correspondingly changing the tracer infusion rate. Another strategy, which was used in

the present study, is a two-compartment model described by Mari (254). This model

describes the glucose system more accurately (than the one-compartment model) under

non-steady-state conditions. R_a was calculated as the sum of three terms: a steady-state

term, a term for the first compartment, and the term for the second compartment. The

principle equations are as follows, where the expression of R_a, calculated at the equally spaced time instants t_0, t_1,…, t_k, t_{k+1}, is determined from the following formulas:

$$R_a(t_k) = (R^*_{inf})(t_k)/SA(t_k) - V_1[C(t_k)dSA(t_k)/dt] / SA(t_k) - V_2k_{22}[SA(t_k)G(t_k) -$$

$$G^*(t_k)]/SA(t_k) \tag{35}$$

$$G(t_{k+1}) = b_1G(t_k) + b_2C(t_k) + b_3C(t_{k+1}) \tag{36}$$

$$G^*(t_{k+1}) = b_1G^*(t_k) + b_2C^*(t_k) + b_3C^*(t_{k+1}) \tag{37}$$

$$V_2 = V_1k_{12}k_{21}/k^2_{22} \tag{38}$$

where t_k and t_{k+1} are time parameters, respectively; $R_a(t_k)$ and $R^*_{inf}(t_k)$ are the rate of appearance calculated with a two-compartment model (mg/kg/min) and tracer infusion rate (dpm/kg/min), respectively; $SA(t_k)$ and $dSA(t_k)$ are specific activity (dpm/mg) and derivative of specific activity (dpm/mg/min), respectively. V_1 and V_2 (ml/kg) are the volumes of the first and second compartments, respectively; $C^*(t_k)$ and $C(t_k)$ are tracer and tracee concentrations, respectively; k_{12}, k_{21}, and k_{22} are constant rate parameters of the first and second compartments, respectively; $G(t_k)$ and $G^*(t_k)$ are variables calculated recursively from tracee and tracer concentrations, respectively; b_1, b_2, and b_3 are coefficients of recursive equations for calculating $G(t_k)$ and $G^*(t_k)$. Canine parameters used for V_1, V_2, and k_{22} in the present studies were those determined by Dobbins *et al*. (255). It has been reported (255) that under non-steady state conditions where specific activity changes dramatically, glucose appearance determined using the two-compartment model is more accurate than the Steele equation (one-compartment model.)

When glucose was infused, endogenous glucose production (endo R_a) was determined by subtracting the glucose infusion rate (GIR), from total glucose production (R_a).

Of note, there are two major assumptions that are made when using the particular isotope dilution method to determine glucose kinetics. First, the labeled and unlabeled glucose molecules are assumed to be metabolized in the same manner. Secondly, the label is assumed to be irreversibly lost (261).

It should also be noted, however, that since both the liver and the kidneys produce glucose, whole body tracer-determined glucose production is slightly higher than the rate of hepatic glucose production. Although net kidney glucose balance in the postabsorptive state is near zero, the kidney has been estimated to contribute 5-15% to whole body glucose production (262).

Nonhepatic Glucose Uptake

Nonhepatic glucose uptake (non-HGU) was calculated over time intervals using the following formula:

Non-HGU = average total glucose infusion between T1 and T2 + ((T1$_{NHGB}$ + T2$_{NHGB}$)/2)

$\qquad$ – glucose mass change in the pool $\hfill$ (39)

where T1 and T2 indicates the time points for which glucose is being measured. The ((T1$_{NHGB}$ + T2$_{NHGB}$)/2) term will be a negative number in the presence of net hepatic

glucose uptake. The glucose mass change in the pool is calculated using the following

equation:

Glucose mass change in the pool = $((([G_A]_{T2} - [G_A]_{T1}) / 100) * ((0.22 * body\ wt\ in\ kg *$

$$1000 * 0.65) / body\ wt\ in\ kg)) / (T2-T1) \tag{40}$$

where $[G_A]$ is the plasma glucose concentration, T1 and T2 are the time points of the

interval, 0.22 represents the volume of extracellular fluid (the volume of distribution) or

22% of the dog's weight (259), and 0.65 represents the fraction of the pool (258).

<div align="center">Ratio Calculations</div>

Whole body insulin sensitivity (Specific Aim III) was calculated as a ratio of GIR

to arterial plasma insulin, with the value of GIR divided by the arterial plasma insulin

level for every time point. Nonhepatic insulin sensitivity (Specific Aim III) was

calculated as a ratio of Non-HGU to arterial plasma insulin, with the value of Non-HGU

divided by the arterial plasma insulin level for every time point. Hepatic insulin

sensitivity (Specific Aim III) was calculated as a ratio of net hepatic glucose uptake to

hepatic sinusoidal plasma insulin levels, with the value of net hepatic glucose uptake

divided by the hepatic sinusoidal plasma insulin level for every time point. Peripheral

plasma insulin to peripheral plasma glucose ratio (Specific Aim IV) was calculated by the

respective peripheral plasma insulin level divided by the peripheral plasma glucose level

at each time point. Hindlimb glucose clearance and nonhepatic glucose clearance to

arterial plasma insulin ratios (Specific Aim IV) were calculated with the respective

clearance levels divided by arterial plasma insulin levels at each time point, or average

insulin level over the time duration, in the case of the nonhepatic ratio.

Statistical Analysis

Data are expressed as means ± standard error (SE). Statistical comparisons for

time course data were made by two-way ANOVA with repeated measures design run on

SigmaStat (SPSS Science, Chicago, IL). *Post hoc* analysis was performed with Student-

Newman-Kuels Method. When only two values were compared, an independent t-test

was used (SigmaStat, SPSS Science, Chicago, IL). For Specific Aim IV, paired t-tests

were used to compare single value measurements. Statistical significance was accepted

at $P < 0.05$.

CHAPTER III

INTRAPORTAL GLP-1 INFUSION INCREASES NONHEPATIC GLUCOSE UTILIZATION WITHOUT CHANGING PANCREATIC HORMONE LEVELS

(Adapted from Johnson *et al., Am J Physiol Endocrinol Metab* 293:E1085-E1091, 2007)

<u>Specific Aim I-Introduction</u>

When GLP-1 is secreted, it enters capillary blood in the gut where it is rapidly degraded by DPP-IV. It then enters the hepatic portal vein blood thereby exposing the liver to a high level of the hormone. The short half-life of injected GLP-1 (1-2 min) reflects its rapid degradation by DPP-IV in plasma and throughout the vascular system (263). Nevertheless, after an orally delivered nutrient load, active GLP-1 levels in blood increase 5-10 fold, with levels in the portal vein being approximately twice those in peripheral blood (47). This creates a situation in which GLP-1Rs in the portal vein are exposed to high levels of GLP-1 postprandially, thus making the portal vein region a likely candidate for a site at which GLP-1 could initiate some of its effects.

The neural circuitry is in place to allow GLP-1 interaction with its receptors in the portal vein wall to trigger a physiologic response (87). In the rat, there was an increase in afferent discharges from the hepatic branch of the vagus nerve when GLP-1 was infused into the hepatic portal vein (96). This same intraportal bolus of GLP-1 also increased efferent discharges from the pancreatic branch of the vagus nerve, an effect that was lost with hepatic vagotomy (96). These data raise the possibility that GLP-1R activation within the portal vein may contribute to the incretin and perhaps other effects of GLP-1.

Interestingly, the afferent neural firing from the hepatoportal region continued to increase for 90 min even when exposure to GLP-1 was brief (96). It is unknown if this increase in firing triggers efferent effects at sites other than the pancreas.

We have previously shown that under clamped hyperglycemic-hyperinsulinemic conditions (two-fold basal, four-times basal, respectively) an intraportal infusion of pharmacological amounts of GLP-1 increases net hepatic and non-hepatic glucose uptake in dogs (48). We have also shown under these same pancreatic and hyperglycemic clamp conditions, that a physiological infusion of GLP-1 into the hepatic portal vein or the hepatic artery for 90 min results in an increase in net hepatic glucose uptake, without a change in non-hepatic glucose uptake (47). Since these studies were conducted in the presence of a pancreatic clamp, it is still not known if intraportal delivery of GLP-1 is of significance to the known incretin effects of GLP-1. Previous studies carried out by others (49; 235; 236) in an attempt to address this question have not incorporated a GLP-1 infusion into the hepatic artery; thus it is not possible to distinguish between effects initiated within the liver, per se, versus the hepatic portal vein. In addition, these studies used a bolus, (235) stepwise infusion, (49) or constant infusion (236) to increase hyperglycemia, unlike the clamp conditions which are used in the current study. Therefore the aim of the present study was to determine whether or not the hepatic portal vein is the initiation site for any of the acute effects of GLP-1.

Results

Plasma glucose levels. In response to glucose infusion, there was an increase in plasma glucose levels in the artery (to 158 ± 1, 156 ± 1, 156 ± 1 mg/dl) and portal vein (to 177 ±

2, 174 ± 1, 176 ± 2 mg/dl) in CON, POR, and HAT, respectively (Figure 3.1A). There were no differences among groups.

Plasma GLP-1 levels. Basal plasma GLP-1 levels were similar regardless of group in the artery (2.5 ± 1.7, 2.8 ± 0.4, 3.7 ± 0.8 pM in CON, POR, and HAT), the hepatic portal vein (2.4 ± 1.7, 2.9 ± 0.4, 3.5 ± 0.7 pM), and hepatic vein (2.3 ± 1.8, 3.2 ± 0.4, 3.9 ± 0.8 pM) (Figure 3.1B). GLP-1 levels in the artery, hepatic portal vein, and hepatic vein (3.5 ± 1.6, 3.7 ± 1.4, 3.5 ± 1.3 pM) did not change in response to the saline infusion. On the other hand, they rose in the artery (27.1 ± 2.4, 23.4 ± 2.5 pM; POR, HAT, respectively), the hepatic portal vein (50.0 ± 4.2, 22.8 ± 3.1 pM), and the hepatic vein (41.9 ± 3.0, 41.6 ± 5.8 pM) in response to infusion of GLP-1 into the hepatic portal vein or hepatic artery, respectively (Figure 3.1B). It should be noted that, regardless of the site of GLP-1 infusion, the GLP-1 levels in peripheral (arterial) and liver sinusoidal (hepatic vein) blood were matched in the two GLP-1 infusion groups. Conversely, hepatic portal vein levels were markedly higher during portal vein GLP-1 infusion (Figure 3.1B).

Hepatic GLP-1 fractional extraction. Due to extremely low levels of GLP-1 at baseline, hepatic fractional extraction of active GLP-1 was only calculated for the portal GLP-1 infusion group. The average hepatic GLP-1 fractional extraction for the POR group was approximately 0.08.

Glucose infusion rates. There was no significant difference in the glucose infusion rate required to maintain the clamp in the saline and hepatic artery GLP-1 infusion groups (6.0 ± 0.5, 6.7 ± 1.0 mg/kg/min, average over final 2 hours) (Figure 3.2). When GLP-1 was given intraportally, on the other hand, significantly more glucose was required (8.5 ±
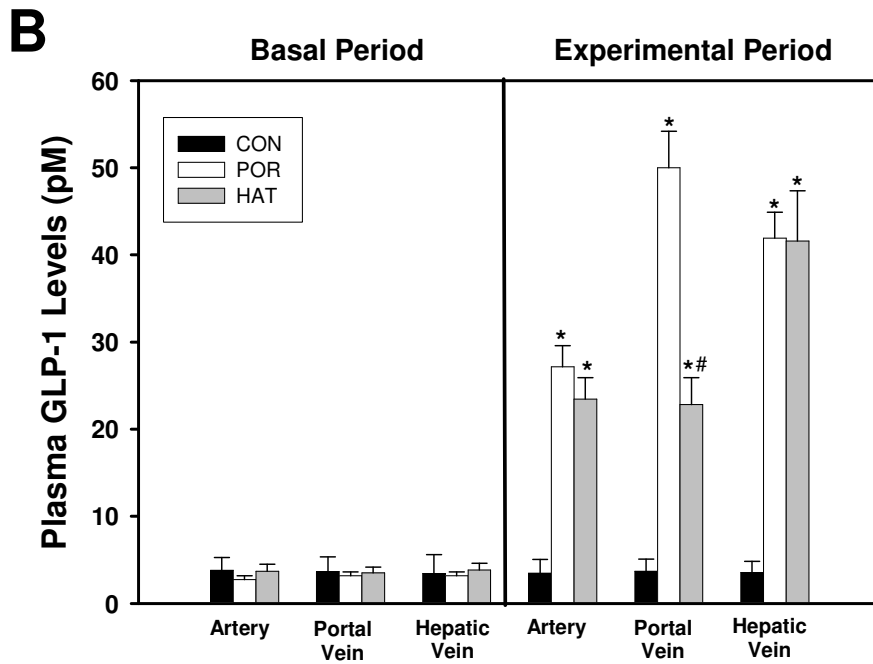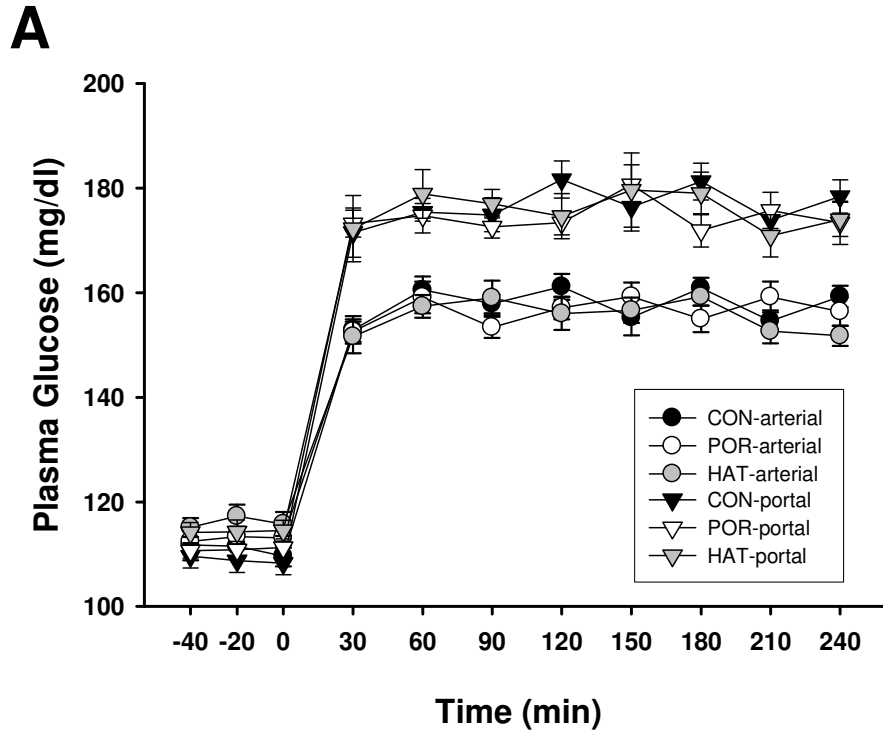
Figure 3.1 *A*: Plasma glucose levels in 42-h fasted conscious dogs were basal initially (-40 to 0 min), but both arterial and portal levels increased significantly ($p < 0.05$) during the experimental period (0 to 240 min) in response to the glucose clamp. There were no significant differences among groups in either the basal or experimental period. Data are expressed as mean ± SE for each time point. *B*: Femoral artery, portal vein, and hepatic vein plasma GLP-1 levels during the basal period (left) and experimental period (right). Data are expressed as mean ± SE. * = $p < 0.05$ versus CON. # = $p < 0.05$ versus POR.
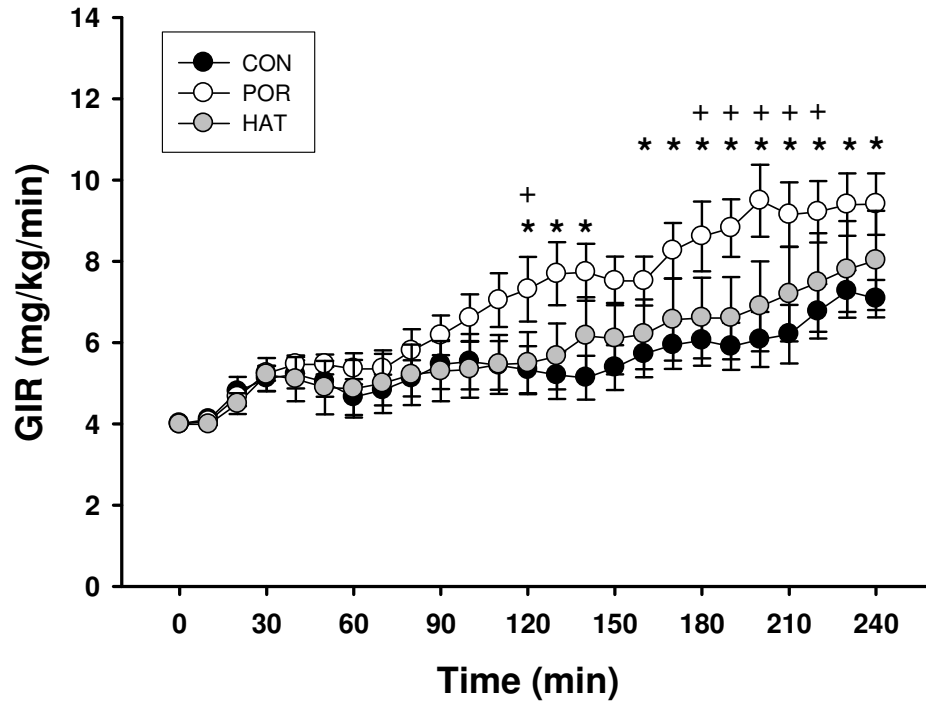
Figure 3.2 Total glucose infusion rate (GIR) during the infusion of saline intraportally, or GLP-1 into the hepatic portal vein or hepatic artery (0 to 240 min). POR required significantly greater GIR than either CON or HAT. Data are expressed as mean ± SE for each time point. $* = p < 0.05$ versus CON. $+ = p < 0.05$ versus HAT.

0.7 mg/kg/min, average over the final 2 hours) than with either saline or hepatic artery GLP-1 infusion (Figure 3.2).

Plasma insulin and glucagon levels. The arterial plasma insulin levels in the basal period were similar (7 ± 1, 5 ± 1, 9 ± 1 µU/ml in CON, POR, and HAT) in the three groups. Likewise, they rose similarly in response to the hyperglycemia brought about during the experimental period (to 24 ± 2, 23 ± 3, 23 ± 3 µU/ml) (Figure 3.3A). Portal plasma insulin levels increased from baseline (26 ± 5, 16 ± 5, 31 ± 9 µU/ml to 82 ± 4, 75 ± 6, 69 ± 8 µU/ml) in response to hyperglycemia (Figure 3.4A). There was no statistical difference in the total AUC in either arterial or portal plasma insulin among groups during the experimental (0 to 240 min) period (Figure 3.3A, 4A insets). The arterial plasma glucagon levels decreased significantly in CON, POR, and HAT during the experimental period (to 23 ± 2, 30 ± 3, 25 ± 2 pg/ml, respectively) from their respective basal values (36 ± 3, 43 ± 4, 36 ± 3 pg/ml) (Figure 3.3B). Portal plasma glucagon levels also decreased from basal (50 ± 6, 51 ± 4, 46 ± 3 pg/ml) during the experimental period (to 27 ± 1, 32 ± 3, 28 ± 2 pg/ml) (Figure 3.4B). There was no statistical difference in the total AUC for either the arterial or portal plasma glucagon during the experimental period (Figure 3.3B, 3.4B insets).

Hepatic blood flow, NHGB and non-HGU. Hepatic artery blood flows during the basal period (7.2 ± 1.0, 5.4 ± 0.4, 4.9 ± 0.7 ml/kg/min in CON, POR, and HAT) and the experimental period (7.9 ± 0.7, 6.8 ± 0.4, 6.4 ± 1.0 ml/kg/min) were not different among the groups. There was also no difference in hepatic portal vein blood flow in either the basal (22.7 ± 2.0, 28.9 ± 2.1, 24.3 ± 1.8 ml/kg/min) or experimental periods (22.7 ± 1.8, 27.9 ± 1.9, 25.1 ± 1.7 ml/kg/min). In the basal state, net hepatic glucose output was
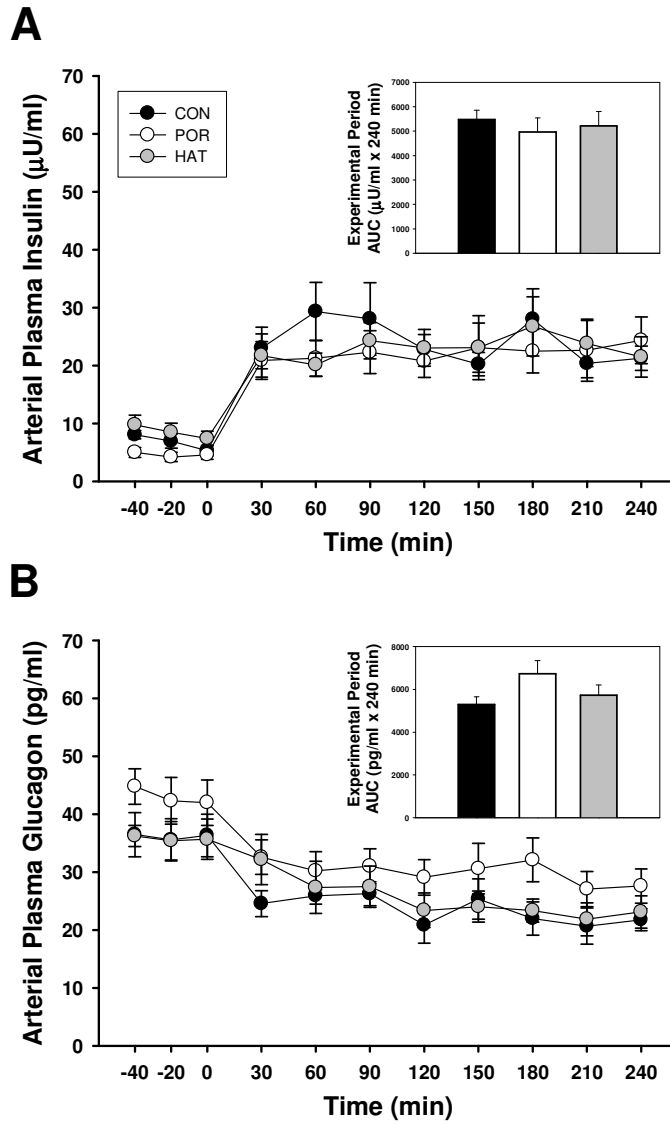
Figure 3.3 *A*: Arterial plasma insulin levels during the basal period (-40 to 0 min) and during the infusion of saline, or GLP-1 into the hepatic portal vein or hepatic artery (0 to 240 min). There was a significant increase in insulin levels in each group during the experimental period when compared to respective basal period values ($p < 0.05$); *inset*: AUC for experimental period. There were no differences among groups upon analysis of the time-course or experimental period AUC. Data are expressed as mean ± SE. *B*: Arterial plasma glucagon levels during the basal period (-40 to 0 min) and during the infusion of saline intraportally, or GLP-1 into the hepatic portal vein or hepatic artery (0 to 240 min); *inset*: AUC during the experimental period. There was a significant decrease in glucagon levels in each group during the experimental period when compared to respective basal period values ($p < 0.05$), but there were no differences among groups upon analysis of the time-course or experimental period AUC. Data are expressed as mean ± SE.
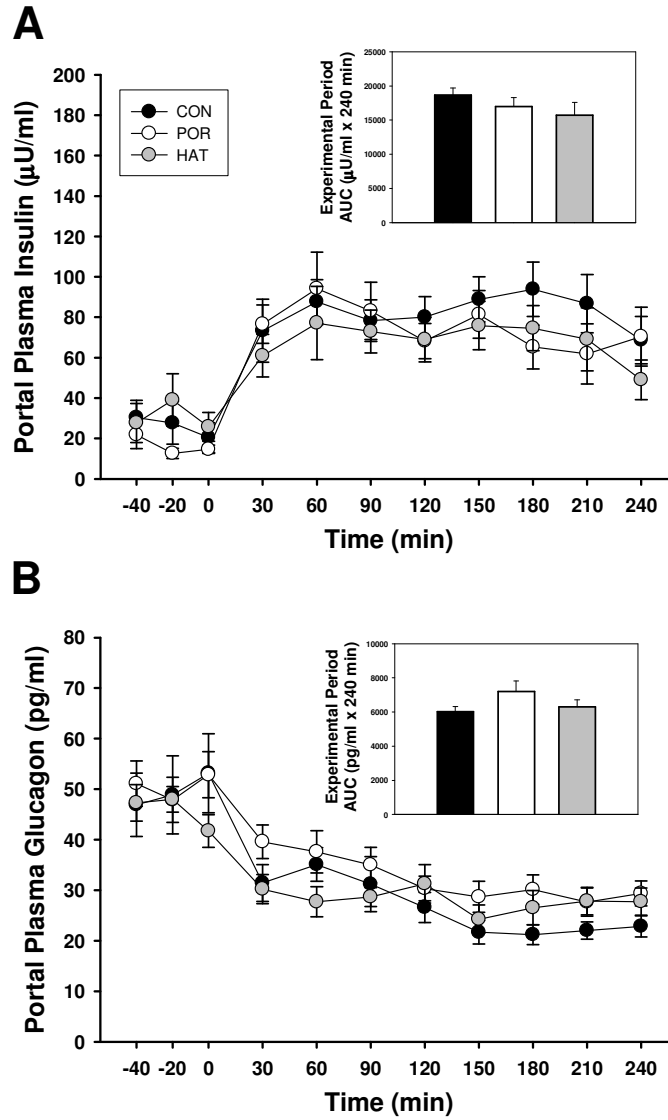
Figure 3.4 *A*: Portal plasma insulin levels during the basal period (-40 to 0 min) and during the infusion of saline, or GLP-1 into the hepatic portal vein or hepatic artery (0 to 240 min). There was a significant increase in insulin levels in each group during the experimental period when compared to respective basal period values ($p < 0.05$); *inset*: AUC for experimental period. There were no differences among groups upon analysis of the time-course or experimental period AUC. Data are expressed as mean ± SE. *B*: Portal plasma glucagon levels during the basal period (-40 to 0 min) and during the infusion of saline intraportally, or GLP-1 into the hepatic portal vein or hepatic artery (0 to 240 min); *inset*: AUC during the experimental period. There was a significant decrease in glucagon levels in each group during the experimental period when compared to respective basal period values ($p < 0.05$), but there were no differences among groups upon analysis of the time-course or experimental period AUC. Data are expressed as mean ± SE.

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similar in all groups (1.6 ± 0.3, 2.0 ± 0.2, 1.6 ± 0.1 mg/kg/min). In response to the

hyperglycemic clamp the liver switched to net glucose uptake, with statistically greater

net hepatic glucose uptake when GLP-1 was given into the hepatic artery (NHGB = -2.4

± 0.4, -3.0 ± 0.4, -3.9 ± 0.4 mg/kg/min in CON, POR, and HAT, respectively, during

final 2 hours) (Figure 3.5A). Non-HGU was not different during saline or hepatic artery

GLP-1 infusion (3.8 ± 0.7, 3.0 ± 0.8 mg/kg/min, respectively, final 2 hours); but it was

significantly greater (5.5 ± 0.8 mg/kg/min, final 2 hours) when GLP-1 was given into the

hepatic portal vein (Figure 3.5B).

Endogenous Glucose Production and Glucose Disposal. Endogenous glucose

production (R_a) was similar among groups during the basal period (2.3 ± 0.2, 2.3 ± 0.2,

2.5 ± 0.1 mg/kg/min in CON, POR, and HAT), and decreased in response to

hyperglycemia during the experimental period (1.0 ± 0.4, 0.5 ± 0.6, 1.0 ± 0.4 mg/kg/min,

average final 2 hours in CON, POR, and HAT) (Figure 3.6A). Glucose disposal (R_d) was

the same among groups during the basal period (2.4 ± 0.2, 2.3 ± 0.1, 2.4 ± 0.1 mg/kg/min

in CON, POR, and HAT) and increased in response to the hyperglycemia (6.7 ± 0.8, 7.8

± 1.1, 7.3 ± 1.0 mg/kg/min, average final 2 hours in CON, POR, and HAT) (Figure

3.6B).

Arterial plasma free-fatty acid. Arterial plasma free-fatty acid levels decreased similarly

in response to glucose infusion (from 994 ± 127, 883 ± 80, 1073 ± 84 μmol/L to 362 ±

111, 168 ± 14, 274 ± 45 μmol/L in CON, POR, and HAT, respectively) in all three

groups.
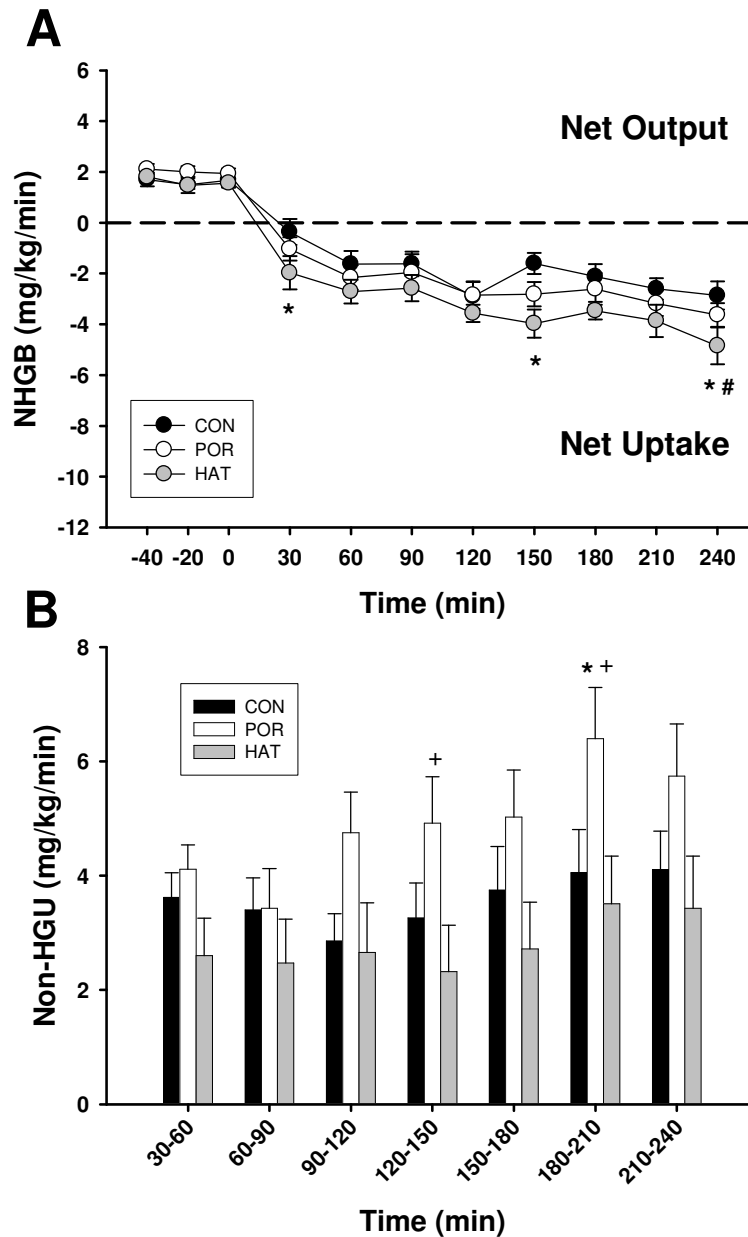
Figure 3.5 *A*: Net hepatic glucose balance (NHGB) during the basal period (-40 to 0 min) and during the infusion of saline, or GLP-1 into the hepatic portal vein or hepatic artery (0 to 240 min). Rates in each group were significantly decreased (p < 0.05) during the infusion of saline, or GLP-1 into the hepatic portal vein or hepatic artery (0 to 240 min), when compared to their respective basal period values. Data are expressed as mean ± SE. * = p < 0.05 versus CON. # = p < 0.05 versus POR. *B*: Non-hepatic glucose uptake (Non-HGU) during the infusion of saline, or GLP-1 into the hepatic portal vein or hepatic artery (30 to 240 min). Data are the average of values over 30 min segments and are expressed as mean ± SE. * = p < 0.05 versus CON. + = p < 0.05 versus HAT.
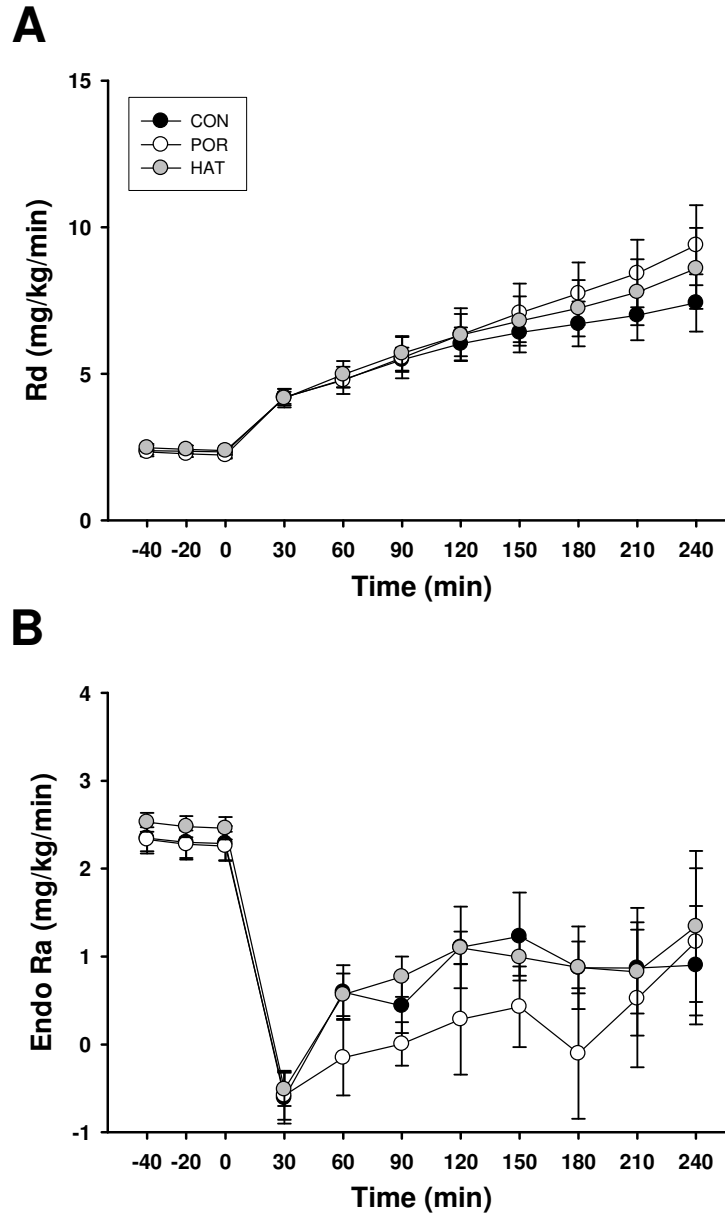
Figure 3.6 *A*: Glucose disposal (R_d) during the basal period (-40 to 0 min) and during the infusion of saline, or GLP-1 into the hepatic portal vein or hepatic artery (0 to 240 min). Data are expressed as mean ± SE. *B*: Endogenous glucose production (R_a) during the basal period (-40 to 0 min) and during the infusion of saline intraportally, or GLP-1 into the hepatic portal vein or hepatic artery (0 to 240 min). Data are expressed as mean ± SE.

Discussion

After a meal, intraportal GLP-1 levels are approximately twice those in peripheral blood (47). Therefore, GLP-1R in the hepatic portal vein are exposed to higher GLP-1 concentrations than receptors in other sites of the body. This makes the hepatic portal vein a potential site for initiation of some of GLP-1's effects. Studies in rats showed that an intraportal injection of GLP-1 resulted in increased afferent impulses from the hepatic branch of the vagus nerves (96). This suggests that GLP-1 can initiate a neural signal within the hepatic portal vein which could result in actions at other sites in the body. In the present study our goal was to determine which, if any, of GLP-1's effects are initiated at hepatic portal vein. The experimental design was such that the action of GLP-1 on gastric emptying and β-cell proliferation were of no consequence. In addition, by including groups in which GLP-1 was infused into either the hepatic artery or the hepatic portal vein, we were able to differentiate between effects resulting from elevations of GLP-1 within the liver versus those within the portal vein. Therefore, we were able to assess the effects of portal vein GLP-1 per se on pancreatic hormone secretion and on the rate of glucose uptake by the liver and nonhepatic tissues.

A significantly greater glucose infusion rate (8.5 ± 0.7 vs. 6.7 ± 1.0 mg/kg/min, average over the final 2 hours) was required to maintain the glucose clamp when GLP-1 was given intraportally than when it was given at the same rate into the hepatic artery (Figure 3.2), despite there being no difference in pancreatic hormone levels (Figures 3.3 & 3.4). This trend was also apparent upon evaluation of glucose disposal (Figure 3.6A). Increased glucose utilization occurred with intraportal infusion of GLP-1, even though the arterial and hepatic vein GLP-1 levels were identical in the two groups (Figure 3.1B);

therefore, the difference in the glucose infusion rate must be attributed to the difference in the hepatic portal vein GLP-1 levels (48.7 ± 4.7 vs. 22.8 ± 3.1 pM). The hepatic portal vein GLP-1 concentrations increased 10-fold when GLP-1 was given into the hepatic artery, versus 20-fold when it was given into the portal vein (Figure 3.1B). The increase (over saline) in the peripheral glucose infusion rate required in the portal GLP-1 infusion group was 3-times greater than the increase in the glucose infusion rate which was required in the hepatic artery infusion group. This difference in glucose infusion rate was slow to develop, reaching a maximum approximately 120 min after the start of GLP-1 infusion. Thereafter the difference between groups was maintained (Figure 3.2).

The delay in the onset of the effect of portally delivered GLP-1 occurred despite the fact that GLP-1 levels were elevated in a square wave fashion. An increase (over saline) in the glucose infusion rate actually occurred as early as 40 min after the start of portal GLP-1 infusion, but it did not reach significance until 120 min (Figure 3.2). The delay in the rise in the glucose infusion rate indicates that the pharmacokinetic time course of GLP-1 differs from the time course of the pharmacodynamic response to its infusion. This was also evident in an earlier study in which a bolus of GLP-1 given into hepatic portal vein of the rat caused an ever increasing rise in afferent neural discharges over time (96). These findings suggest that certain effects of GLP-1 may last for a longer period of time than the increment during which GLP-1 blood level is increased. It has been shown that postprandial GLP-1 levels remain elevated for as long as 3 hours in the human (35) and 6 hours in the dog (unpublished data). The present data suggest that the impact of the rise in GLP-1 might go on for an even longer period of time. The

mechanistic explanation for this slow onset of action may relate to persistent changes in neural firing or the time required for the synthesis of regulatory proteins.

Given that intraportal infusion of GLP-1 increased whole body glucose disposal in the absence of a change in plasma insulin, the question thus arises as to which tissues were responsible for the increase. By placing catheters across the liver, one can separate whole body glucose uptake into its hepatic and nonhepatic components. There was only a small increase (3.0 ± 0.4 vs. 2.4 ± 0.4 mg/kg/min, not significant) in net hepatic glucose uptake (Figure 3.5A), and small decrease in endogenous glucose production (1.0 ± 0.4 vs. 0.5 ± 0.6 mg/kg/min, not significant) (Figure 3.6B), in the animals that received intraportal GLP-1 versus saline. As a result it can be concluded that the increase in whole body glucose uptake seen in those animals was primarily due to an increase in nonhepatic glucose uptake. This is confirmed by our observation that the calculated rate of nonhepatic glucose uptake was significantly increased in the presence of intraportal as opposed to hepatic artery GLP-1 infusion (Figure 3.5B) (3.0 ± 0.8 versus 5.5 ± 0.8 mg/kg/min, respectively during the final 2 hours of the study). The combined effects of hepatic and nonhepatic glucose uptake are in agreement with the rate of glucose utilization (Figure 3.6). It is also in agreement with previously reported studies which indicated that portal vein GLP-1 receptors regulate nonhepatic glucose uptake in the mouse in the presence of an intraportal glucose infusion (236; 264). A previous study conducted in our lab showed that a physiological increase in GLP-1 resulting from intraportal infusion of the peptide at the same rate as that used here, when brought about with the pancreatic hormones clamped at levels similar to those observed in the current study, caused a small increase in net hepatic glucose uptake (approximately 0.8

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mg/kg/min) much like in the present study, but, contrary to the present results GLP-1

infusion in the clamp studies had no effect on non-hepatic glucose uptake (47). However,

the duration of the intraportal GLP-1 infusion in the previous study was only 90 min (47),

perhaps explaining the fact that we did not see a significant increase in nonhepatic

glucose uptake (i.e. in the present study the effect did not become significant until 120

min). In addition, in our previous study, glucose was clamped using only a peripheral

infusion (i.e. no portal glucose delivery). It has been clearly shown that when glucose is

delivered intraportally, it decreases afferent vagal firing (265), and causes an increase in

net hepatic glucose uptake and a decrease in nonhepatic glucose uptake (176). Portal

delivery of GLP-1 increases vagal afferent firing (96); thus it is possible that in the

presence of portal glucose delivery GLP-1 can bring about effects which would not be

observed in the absence of portal glucose delivery.

Taking the results from our previous study (47) and the current study together it

would appear that a physiological increase in GLP-1 secretion can bring about two

effects, each independent of the actions of GLP-1 on the endocrine pancreas. First, GLP-

1 can have a direct, but modest, effect on the liver per se to increase net hepatic glucose

uptake. This is seen independently of whether the rise in liver sinusoidal GLP-1 results

from input via the hepatic artery or the hepatic portal vein (47). It can best be seen by

examining the hepatic artery GLP-1 infusion data (i.e. those without the added action of

portal vein GLP-1 signaling). In our earlier study, net hepatic glucose uptake increased

by approximately 1.0 mg/kg/min (47) in response to hepatic artery GLP-1 infusion, while

in the present study it increased by 1.5 mg/kg/min (2.4 ± 0.4 vs. 3.9 ± 0.4 mg/kg/min).

This was despite the fact that there were no differences in plasma insulin or glucagon

level in the presence of GLP-1, compared to the control, in either study. The second effect of GLP-1 relates to its delivery into the portal vein. There it tends to decrease net hepatic glucose uptake (from 3.9 ± 0.4 to 3.0 ± 0.4 mg/kg/min), and to increase nonhepatic glucose uptake (from 3.0 ± 0.8 to 5.5 ± 0.8 mg/kg/min). The question then arises as to why a 20-fold rise in portal vein GLP-1 would bring about this effect and a 10 fold increase would not. This could have resulted from a threshold effect such that a 10 fold increase simply did not bring about a big enough change for us to detect. Alternatively, the portal vein concentration difference in GLP-1 may be detected and bring about a unique response.

It has been well established that exogenously infused GLP-1 acts as an incretin in both healthy humans and those with type 2 diabetes (40). As noted above, however, in the current studies there was no difference in arterial or portal plasma insulin levels in the presence or absence of GLP-1 infusion regardless of whether the peptide was given intraportally or via the hepatic artery (Figure 3.3A). This agrees with earlier data indicating that dogs which received a systemic infusion of glucose to simulate postprandial peripheral glucose levels showed no change in insulin levels when a peripheral GLP-1 infusion was added to create a physiological increase in GLP-1 levels (49).

Postprandial increases in peripheral plasma GLP-1 levels in the human and dog are very similar (both reaching levels of approximately 10-15 pM); however, we did not observe greater insulin levels in the presence versus the absence of GLP-1 in the current study, despite the fact that our GLP-1 levels were designed to match postprandial levels. There are several possible explanations for our failure to observe an incretin effect of

GLP-1. There is some evidence that the GLP-1 clearance in the human and the dog may be different. It has been shown that an infusion rate of approximately 0.3 pmol/kg/min (266) is required to create a postprandial rise in total peripheral GLP-1 levels in the human, while the dog requires 3 to 4 times that rate to simulate post meal levels of active GLP-1 (47). This suggests greater clearance of GLP-1 in the dog, such that for a given infusion rate, the plasma levels would be lower in the dog; therefore, the 1 pmol/kg/min infusion rate used here might result in significantly higher GLP-1 levels in humans, which could induce significantly greater effects at the β-cell.

On the other hand, there may be a species difference in β-cell sensitivity to GLP-1, because it has been shown that in humans an infusion of 0.15 pmol/kg/min, in the presence of ~180 mg/dl plasma glucose levels, can augment glucose stimulated insulin secretion (266), while in the current study an infusion rate of 1 pmol/kg/min in the presence of ~160 mg/dl plasma glucose did not change plasma insulin levels. This raises the question of whether endogenously released GLP-1 acts as an incretin hormone in the dog. It could still do so if endogenously released GLP-1 initiates an incretin signal upstream from our infusion site, in closer proximity to the L-cells from which it is released. It is known that a large portion of GLP-1 is degraded in the gut by DPP-IV in the brush-border membrane (267), prior to its reaching the hepatic portal vein; therefore, we may not have observed an increase in insulin in the presence of GLP-1 due to the fact that active GLP-1 levels at the gut were not high enough to induce an incretin effect.

The data presented here also indicate that GLP-1 produced no additional suppression of the plasma glucagon level in response to hyperglycemia (Figures 3.3B and 3.4B). Our data thus support the concept that GLP-1 infusion decreases plasma

glucagon only in the presence of inappropriately elevated levels of the hormone, as previously observed in patients with diabetes (40; 209).

As previously mentioned, an advantage to our model is the ability to determine substrate balance across the liver. We were able to determine that approximately 8% of active GLP-1 is degraded as it traverses the liver in the healthy dog. This degradation represents a combination of GLP-1 that is degraded by the liver, per se, and the amount that is exposed to and degraded by DPP-IV in plasma during hepatic transit. The measured levels of GLP-1 in the hepatic portal vein were slightly below the value predicted given portal vein plasma flow and the GLP-1 infusion rate. This is undoubtedly the result of imperfect mixing of the infusate in the portal blood. Were recovery of GLP-1 to have been perfect, the portal vein GLP-1 levels would have been modestly higher and the amount of GLP-1 lost on transit through the liver could have been 18%. Thus, it would appear that less than 20% of active GLP-1 is removed from the blood as it traverses the liver.

In conclusion, the current study shows that delivery of a physiological amount of GLP-1 into the hepatic portal vein (but not the hepatic artery) increased whole body glucose uptake in the absence of an effect on plasma insulin or glucagon levels. This increase was primarily due to an augmentation of nonhepatic glucose uptake. At this point, the mechanism by which this effect comes about is unclear. Nevertheless, the current results further support the concept that the non-incretin effects of GLP-1 are also important in its regulation of glucose metabolism *in vivo*.

CHAPTER IV

INTRAPORTALLY DELIVERED GLUCOSE IS REQUIRED FOR INTRAPORTAL GLP-1 INFUSION TO INCREASE NONHEPATIC GLUCOSE UTILIZATION

(Adapted from Johnson *et al., Am J Physiol Endocrinol Metab* 294:E380-E384, 2008)

Specific Aim II-Introduction

After a meal, glucagon-like peptide-1 (GLP-1) is secreted from the L cell in the gut such that its level in the hepatic portal vein is approximately twice that in peripheral blood (47). Likewise, at the same time postprandial glucose levels are higher in hepatic portal vein blood than in the artery, due to absorption of the meal. In Specific Aim I, in the presence of intraportal glucose delivery, a physiological increase of GLP-1 in the hepatic portal vein increased nonhepatic glucose uptake via a mechanism independent of changes in pancreatic hormone secretion (268). This increase in nonhepatic glucose uptake did not occur when GLP-1 was infused at the same rate through the hepatic artery, even though infusion at this site elevated GLP-1 concentrations in the liver and peripheral blood to the same levels as those seen in the presence of intraportal GLP-1 infusion (268). It can be concluded, therefore, that the elevation of GLP-1 in the hepatic portal vein was itself responsible for the increase in nonhepatic glucose uptake.

In addition to showing that GLP-1 delivery into the hepatic portal vein results in changes in glucose utilization, our lab (239; 269-272) and others (264; 273; 274) have assembled evidence that intraportal delivery of glucose per se alters the distribution of a glucose load in the body. When compared to peripheral glucose delivery, intraportal

delivery of glucose results in greater net hepatic glucose uptake even when the liver is presented with equal hepatic glucose loads and sinusoidal insulin levels (269). In addition, we have shown that intraportal delivery of glucose decreases nonhepatic glucose uptake. Thus, portal glucose delivery results in a preferential deposition of glucose in the liver (239; 271; 272). It is believed that these unique effects of portal glucose delivery are mediated by neural signals initiating within the hepato-portal region (275; 276).

Firing of afferent vagal nerve fibers originating in the hepatic portal region decreases upon initiation of an intraportal glucose infusion, thus suggesting that the portal glucose signal is neurally mediated (265; 277). In contrast to glucose, intraportal GLP-1 delivery increases neural firing from the region (96; 278); therefore, both glucose and GLP-1 have the ability to initiate effects via neural changes within the hepatic portal vein. It has been suggested that a negative arterial-hepatoportal gradient for both GLP-1 and glucose levels must be present for GLP-1 to exert its effect on glucose utilization (49; 236; 268). The aim of the present study was to test this hypothesis.

Results

For ease of comparison, results from Specific Aim I, in which hyperglycemia was induced by a combination of intraportal (4 mg/kg/min) and peripheral glucose (variable as needed) infusions have been presented here at PoGlu-SAL and PoGlu-GLP-1. Results from the current study, in which only peripheral glucose infusion was used, are referred to as PeGlu-SAL and PeGlu-GLP-1 in the text.

Plasma glucose levels. In response to a combination of portal and peripheral glucose

infusions, there was an increase in plasma glucose levels in the artery (to 158 ± 1 and 156

± 1 mg/dl) and portal vein (to 177 ± 2 and 174 ± 1 mg/dl) in both PoGlu-SAL and PoGlu-

GLP-1, respectively (Figure 4.1A). The animals that received only peripheral glucose

infusion experienced increases in plasma glucose levels in the artery (to 159 ± 1 and 162

± 1 mg/dl) and hepatic portal vein (to 159 ± 1 and 162 ± 1 mg/dl in PeGlu-SAL and

PeGlu-GLP-1, respectively) that were of similar magnitude (Figure 4.1B).

Plasma GLP-1 levels. Basal plasma GLP-1 levels were similar regardless of group in

both arterial and hepatic portal vein plasma (Figure 4.2). GLP-1 levels in the artery and

hepatic portal vein did not change in response to the saline infusion. On the other hand,

in response to intraportal GLP-1 infusion they rose in the artery (27.1 ± 2.4 and 29.4 ±

1.5 pM; PoGlu-GLP-1 and PeGlu-GLP-1, respectively) and in the hepatic portal vein

(50.0 ± 4.2 and 57.7 ± 4.5 pM) (Figure 4.2), with no difference between groups in either

vessel.

Glucose infusion rates. Intraportal GLP-1 infusion caused a significantly greater

increase ($p < 0.05$) in the glucose infusion rate in the presence of an intraportal glucose

infusion (8.5 ± 0.7 vs. 6.0 ± 0.5 mg/kg/min, average over final 2 hours; PoGlu-GLP-1 vs.

PoGlu-SAL) (Figure 4.3A) than in its absence (6.4 ± 1.2 vs. 6.1 ± 1.0 mg/kg/min,

average over final 2 hours; PeGlu-GLP-1 vs. PeGlu-SAL) (Figure 4.3B).

Plasma insulin and glucagon levels. In the basal period, arterial and hepatic portal vein

plasma insulin levels were similar among all groups (Figure 4.4). Likewise, they rose

similarly in response to the hyperglycemia brought about during the experimental period

(to 24 ± 2, 23 ± 3, 24 ± 1, 28 ± 5 µU/ml of arterial plasma, and to 82 ± 4, 75 ± 6, 81 ± 12,
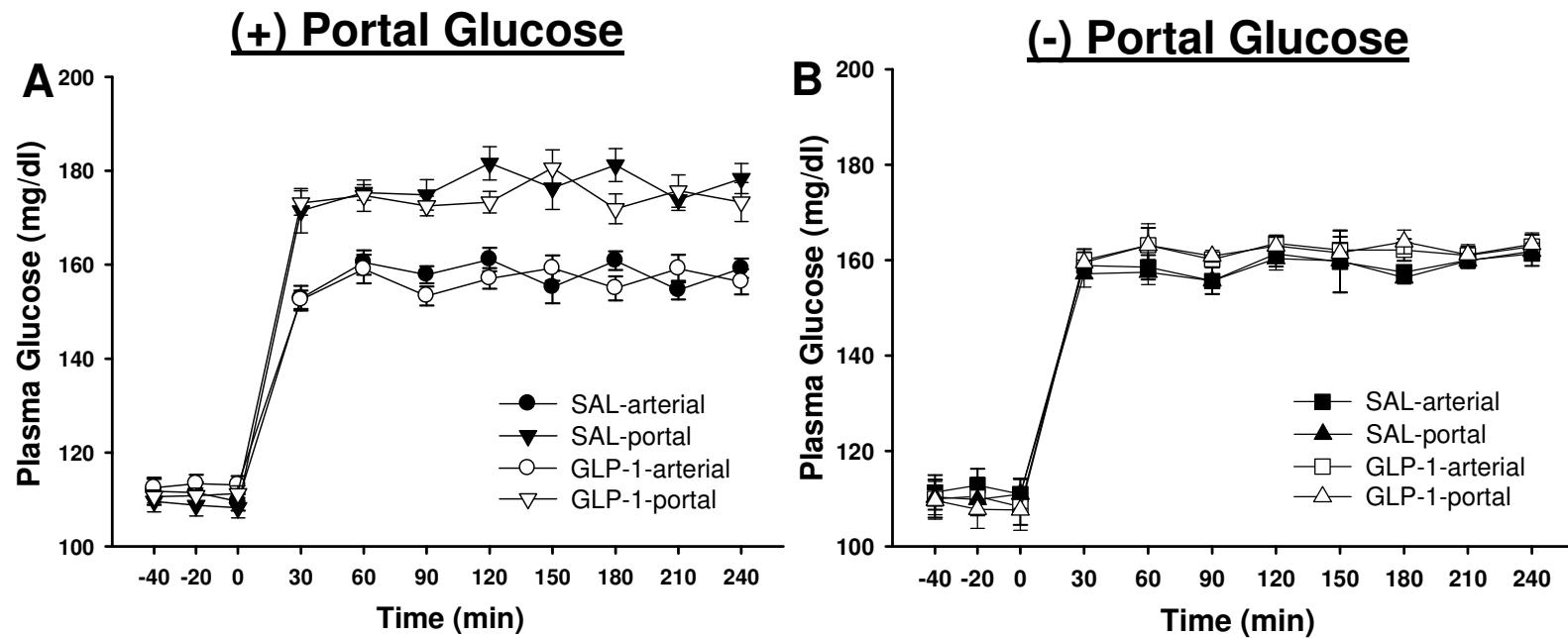
Figure 4.1 Plasma glucose levels in 42-h fasted conscious dogs. *A*: Arterial and portal plasma glucose levels for dogs that received a combination of intraportal and peripheral glucose, in addition to either intraportal GLP-1 or saline (SAL) from Specific Aim I. Levels were basal initially (-40 to 0 min), but both arterial and portal levels increased significantly ($p < 0.05$) during the experimental period (0 to 240 min) in response to the glucose clamp. There were no significant differences among groups in either the basal or experimental period. *B*: Arterial and portal plasma glucose levels for dogs that received a peripheral glucose infusion, in addition to intraportal GLP-1 or saline (SAL). Levels were basal initially (-40 to 0 min), but both arterial and portal levels increased significantly ($p < 0.05$) during the experimental period (0 to 240 min) in response to the glucose clamp. There were no significant differences among groups in either the basal or experimental period. Data are expressed as mean ± SE.
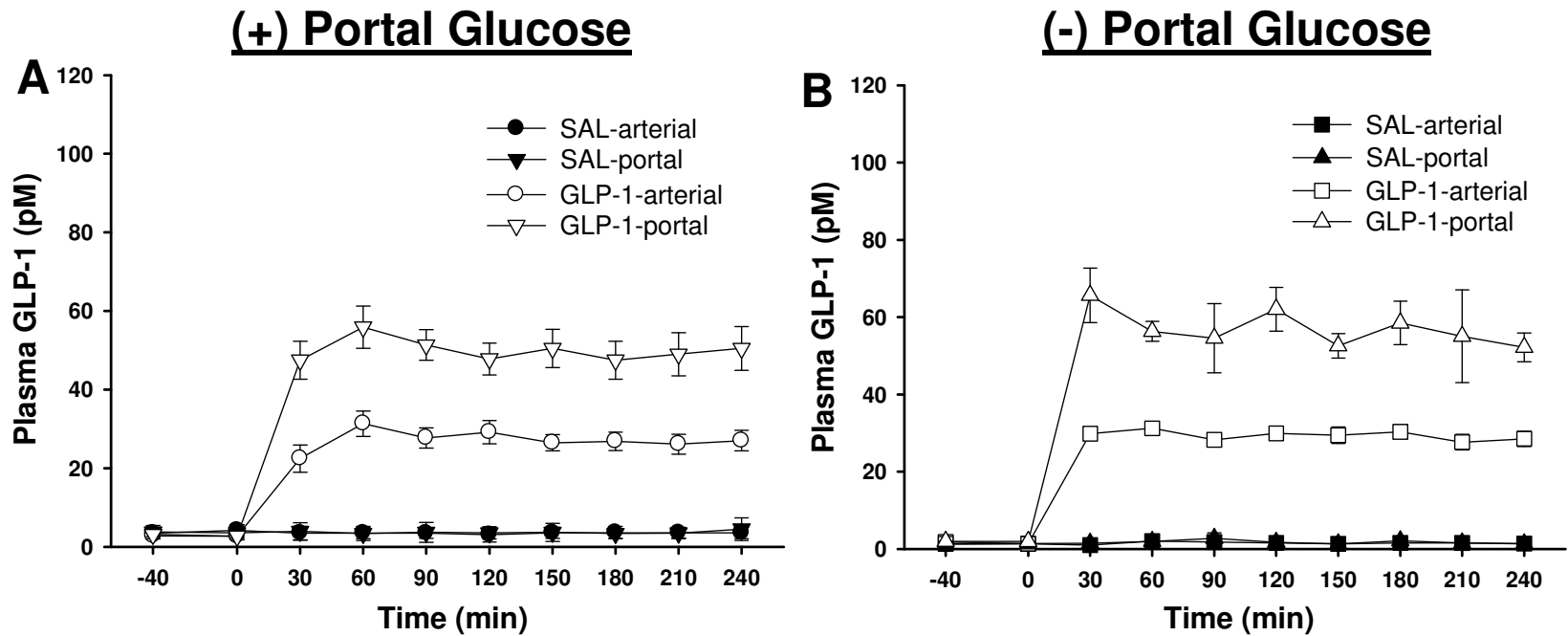
Figure 4.2 Plasma GLP-1 levels. *A*: Arterial and portal plasma GLP-1 levels for animals that received a combination of intraportal and peripheral glucose, in addition to either intraportal GLP-1 or saline (SAL) from Specific Aim I. *B*: Arterial and portal plasma GLP-1 levels for dogs that received a peripheral glucose infusion, in addition to either intraportal GLP-1 or saline. In the animals that received the GLP-1 infusion, GLP-1 levels were basal initially (-40 to 0 min), but both arterial and portal levels increased significantly ($p < 0.05$) during the experimental period (0 to 240 min). Levels remained unchanged in SAL. Data are expressed as mean ± SE.
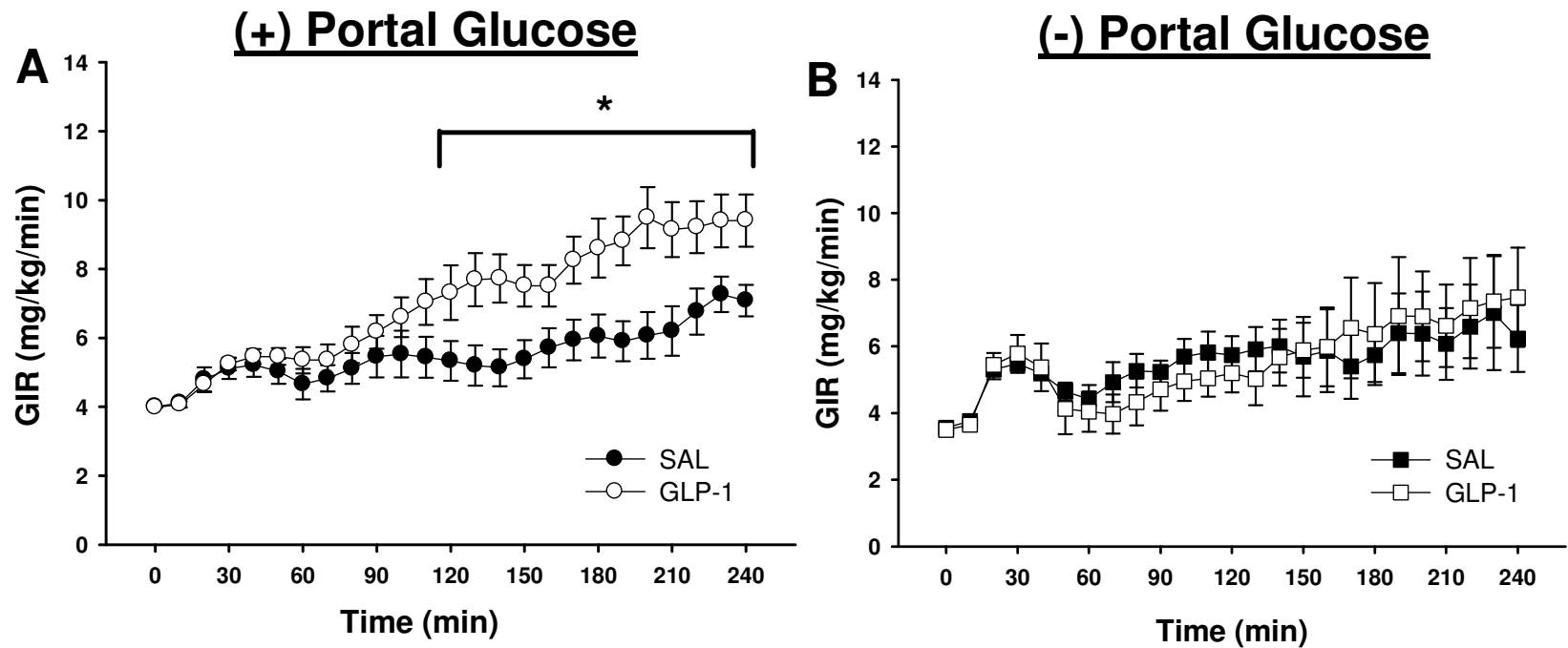
Figure 4.3 Total glucose infusion rate (GIR) during the infusion of intraportal saline (SAL) or GLP-1 into the hepatic portal vein (0 to 240 min). *A*: GIR for animals that received a combination of intraportal and peripheral glucose from Specific Aim I. Data are expressed as mean ± SE for each time point. Animals that received GLP-1 intraportally had a significantly greater GIR than SAL. * = $p < 0.05$ versus SAL. *B*: GIR for animals that received peripheral glucose alone. There was no statistical difference between groups. Data are expressed as mean ± SE for each time point.
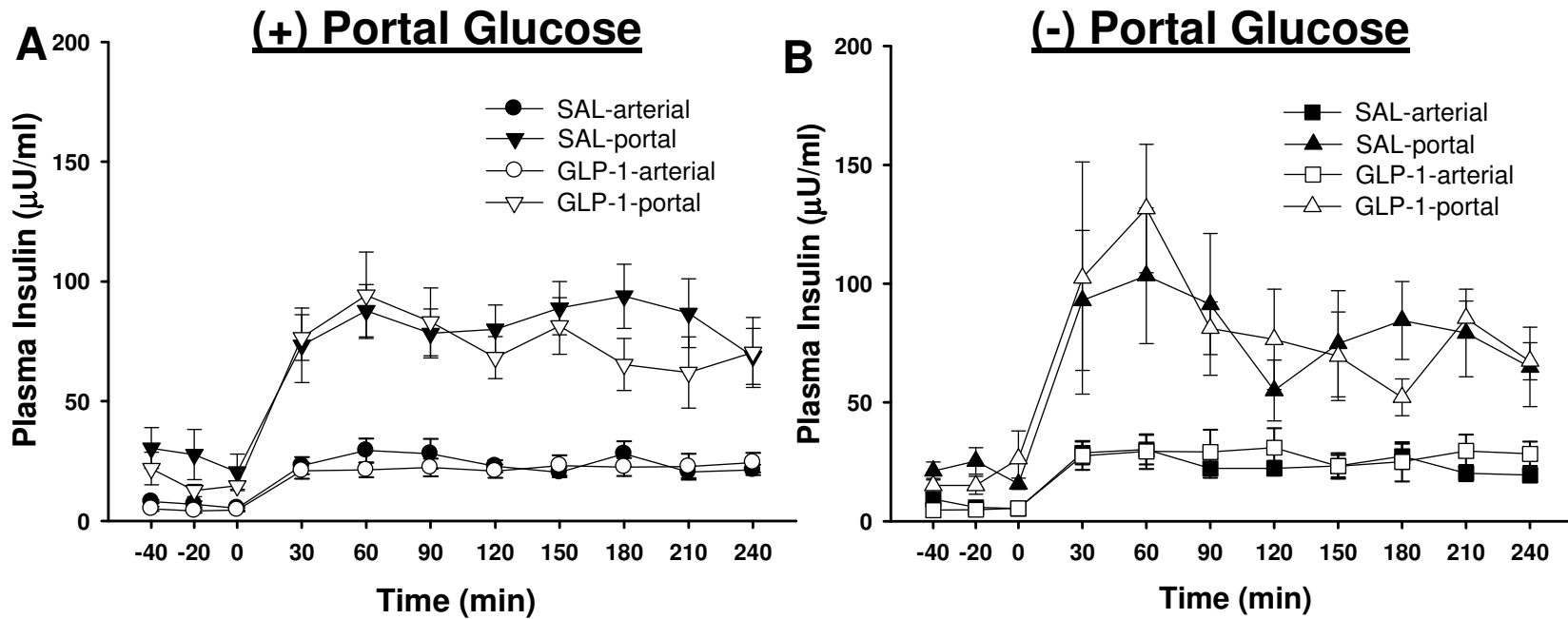
Figure 4.4 Plasma insulin levels. *A*: Arterial and portal plasma insulin levels for animals that received a combination of intraportal and peripheral glucose, in addition to either intraportal GLP-1 or saline (SAL) from Specific Aim I. Levels were basal initially (-40 to 0 min), but both arterial and portal levels increased significantly ($p < 0.05$) during the experimental period (0 to 240 min) in response to the glucose clamp. *B*: Arterial and portal plasma insulin levels for dogs that received a peripheral glucose infusion, in addition to either intraportal GLP-1 or saline. Levels were basal initially (-40 to 0 min), but both arterial and portal levels increased significantly ($p < 0.05$) during the experimental period (0 to 240 min) in response to the glucose clamp. Data are expressed as mean $\pm$ SE.

86 ± 10 µU/ml of hepatic portal vein plasma in PoGlu-SAL, PoGlu-GLP-1, PeGlu-SAL, and PeGlu-GLP-1, respectively) (Figure 4.4). In the basal period, sinusoidal plasma glucagon levels were similar among all groups (Figure 4.5). Likewise, they declined during the experimental period in a similar manner in all groups (to 25 ± 2, 32 ± 3, 22 ± 5, 22 ± 5 pg/ml in PoGlu-SAL, PoGlu-GLP-1, PeGlu-SAL, and PeGlu-GLP-1, respectively).

Hepatic blood flow, HGL, NHGB and non-HGU. Hepatic arterial blood flow during the basal period and the experimental period were similar among the groups, as were the hepatic portal vein blood flows. The hepatic glucose load was approximately 30% greater in those groups that received intraportal glucose delivery (38.6 ± 2.5 and 41.7 ± 2.3 mg/kg/min, average over experimental period; PoGlu-SAL and PoGlu-GLP-1, respectively) than in those that did not (30.1 ± 2.4 and 31.8 ± 2.1 mg/kg/min, average over experimental period; PeGlu-SAL and PeGlu-GLP-1, respectively). In the basal state, net hepatic glucose output was similar in all groups (1.6 ± 0.3, 2.0 ± 0.2, 1.5 ± 0.2, 1.3 ± 0.1 mg/kg/min; PoGlu-SAL, PoGlu-GLP-1, PeGlu-SAL, and PeGlu-GLP-1, respectively). In response to the hyperglycemic clamp the liver switched to net glucose uptake in all groups; however, hepatic portal vein glucose infusion was associated with greater net hepatic glucose uptake than saline infusion (NHGB = -2.4 ± 0.4 and -3.0 ± 0.4 mg/kg/min; PoGlu-SAL and PoGlu-GLP-1, respectively, during final 2 hours vs. -1.2 ± 0.1 and -1.6 ± 0.2 mg/kg/min; PeGlu-SAL and PeGlu-GLP-1, respectively, during final 2 hours) (Figure 4.6A, 4.6B). In the presence of portal and peripheral glucose infusions, non-HGU was significantly greater in the presence of intraportal GLP-1 (5.5 ± 0.8 mg/kg/min, final 2 hours) when compared to saline infusion (3.8 ± 0.7 mg/kg/min, final 2
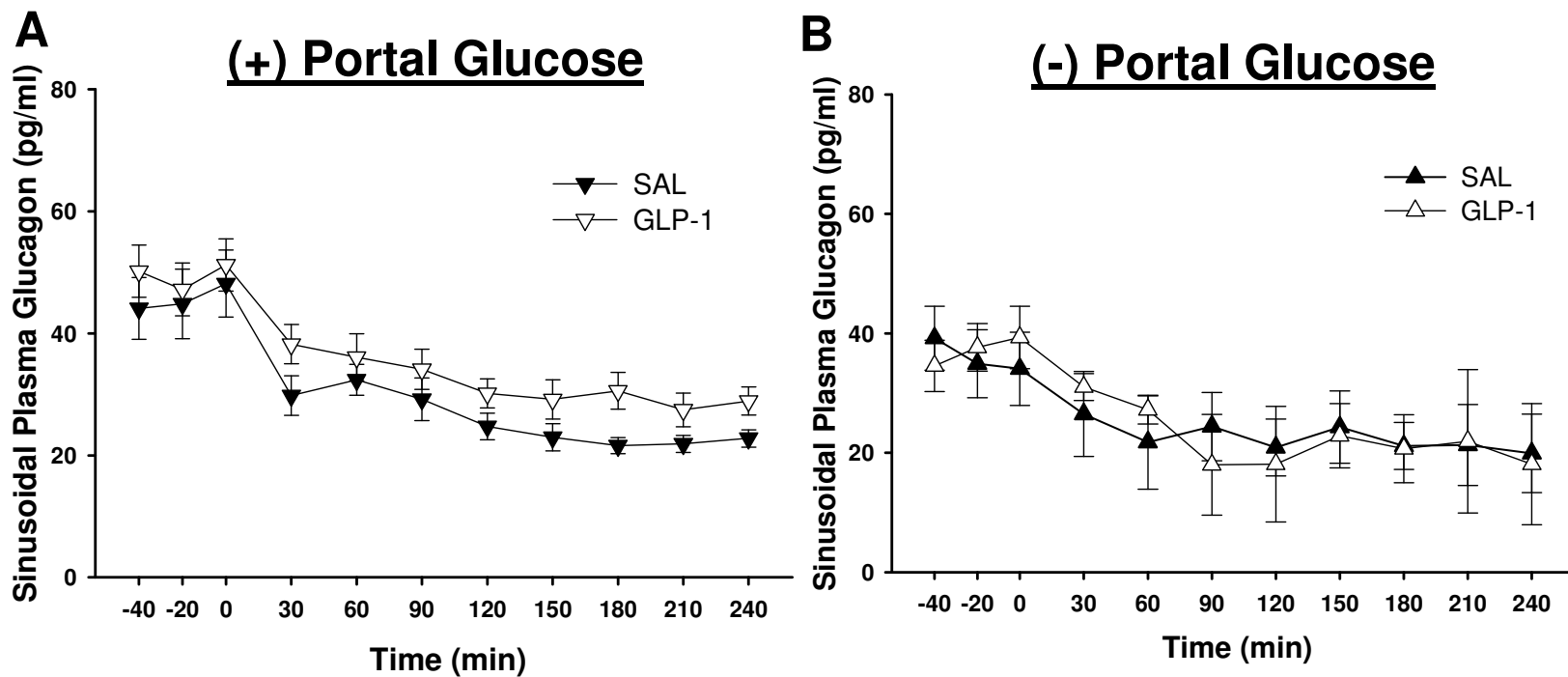
Figure 4.5 Sinusoidal plasma glucagon levels. *A*: Sinusoidal plasma glucagon levels for animals that received a combination of intraportal and peripheral glucose, in addition to either intraportal GLP-1 or saline (SAL) from Specific Aim I. *B*: Sinusoidal plasma glucagon levels for animals that received peripheral glucose infusion, in addition to either intraportal GLP-1 or saline. There was a significant decrease sinusoidal glucagon levels in all groups during the experimental period when compared to respective basal period values ($p < 0.05$). Data are expressed as mean ± SE.
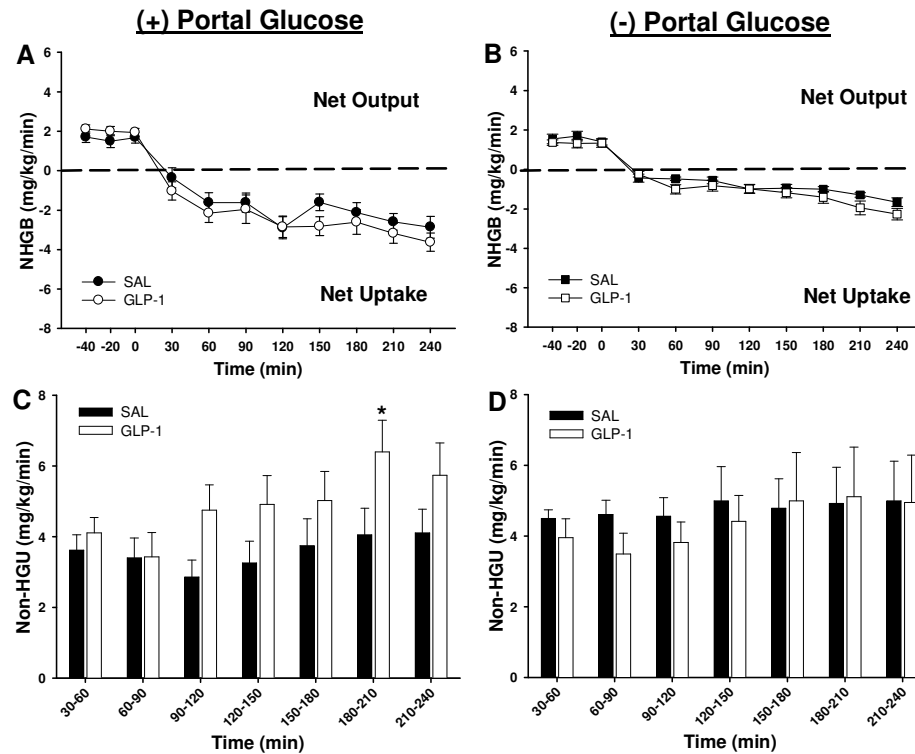
Figure 4.6 Glucose production and utilization. *A*: Net hepatic glucose balance (NHGB) during the basal period (-40 to 0 min) and experimental period (0 to 240 min) in animals that received intraportal and peripheral glucose, in addition to either intraportal GLP-1 or saline (SAL) from Specific Aim I. There was no significant difference between groups. *B*: NHGB in animals that received a peripheral glucose infusion, in addition to either intraportal GLP-1 or saline (SAL). There was no significant difference between groups. *C*: Non-hepatic glucose uptake (Non-HGU) during the infusion of intraportal saline (SAL) or GLP-1 during the experimental period (30 to 240 min) in animals that received both intraportal and peripheral glucose infusions from Specific Aim I. Animals that received GLP-1 had significantly greater ($p < 0.05$) Non-HGU. * = $p < 0.05$ versus SAL. *D*: Non-HGU during the infusion of intraportal saline or GLP-1 during the experimental period (30 to 240 min) in animals that received only a peripheral glucose infusion. There was no significant difference between groups. Data are expressed as mean ± SE.

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hours) (Figure 4.6C). There was no effect of GLP-1 on non-HGU (4.9 ± 1.2 and 4.9 ± 1.0 mg/kg/min, final 2 hours; PeGlu-GLP-1 and PeGlu-SAL) when glucose was supplied solely by peripheral glucose infusion (Figure 4.6D).

Discussion

After a meal, the increases in both GLP-1 (47) and glucose levels in the hepatic portal vein are significantly greater than in the peripheral blood. It has been suggested that this elevation of both GLP-1 and glucose must exist for a physiological increase of GLP-1 to exert its effect on glucose utilization (49; 236; 268). In Specific Aim I, in the presence of hepatic portal vein glucose infusion, a physiological increase of GLP-1 in the hepatic portal vein stimulated nonhepatic glucose uptake via a mechanism independent of changes in pancreatic hormone secretion (268). The results from the present study indicate that, in the absence of a hepatic portal vein glucose infusion, portal vein GLP-1 infusion does not bring about such an effect. In the presence of a portal glucose infusion, the glucose infusion rate required to clamp the glucose at 160 mg/dl was significantly greater when GLP-1 was given than when it was not (8.5 ± 0.7 mg/kg/min vs. 6.0 ± 0.5 mg/kg/min, average over the final 2 hours; PoGlu-GLP-1 vs. PoGlu-SAL). In the absence of portal glucose infusion there was no effect of GLP-1 (6.4 ± 1.2 mg/kg/min vs. 6.1 ± 1.0 mg/kg/min, average over final 2 hours; PeGlu-GLP-1 and PeGlu-SAL) on the glucose infusion rate (Figure 4.3). The failure of the glucose infusion rate to rise is explained by the absence of an increase in nonhepatic glucose uptake (4.9 ± 1.2 and 4.9 ± 1.0 mg/kg/min, final 2 hours; PeGlu-GLP-1 and PeGlu-SAL). In the presence of a portal glucose infusion, nonhepatic glucose uptake was significantly greater when GLP-1 was

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given intraportally than when it was not (5.5 ± 0.8 vs. 3.8 ± 0.7 mg/kg/min, final 2 hours; PoGlu-GLP-1 vs. PoGlu-SAL) (Figure 4.6C, 4.6D).

The question thus arises as to the mechanism by which a physiological increase in hepatic portal vein GLP-1 levels might bring about increased whole body glucose uptake only in the presence of intraportal glucose delivery. Intraportal glucose delivery decreases vagal neural firing by afferent fibers originating in the hepatic portal vein (265; 277), and it has been shown that these nerves must be intact for the portal glucose signal to initiate its effects (275; 276). Portally delivered GLP-1 on the other hand, increases neural firing in afferents originating in the hepato-portal region (96; 278). It has been clearly shown that the portal glucose signal decreases nonhepatic glucose uptake (271; 272). Thus if GLP-1 were to override the impact of portally delivered glucose on vagal afferent firing to nonhepatic tissue, one would predict an increase in nonhepatic glucose uptake. Since our earlier work has suggested that the effect of the portal signal occurs in muscle, it seems likely that this is the site of the GLP-1 induced effect (272).

Our earlier data by Dardevet et. al (47) showed that intraportal infusion of GLP-1 (1 pmol/kg/min) in the presence of hyperglycemia induced by peripheral glucose infusion and a pancreatic hormone clamp, resulted in a small increase in net hepatic glucose uptake (approximately 0.8 mg/kg/min), but no change in nonhepatic glucose uptake. This agrees with data from the current study, in which animals that received glucose only via the peripheral route tended to have slightly greater net hepatic glucose uptake when GLP-1 was given intraportally than when it was not (NHGB= -1.6 ± 0.2 vs. -1.2 ± 0.1 mg/kg/min, during final 2 hours, not significant, p=0.16; PeGlu-GLP-1 vs. PeGlu-SAL) (Figure 4.6B). As noted in Specific Aim I, and as recapitulated here, in the presence of

intraportal glucose infusion, GLP-1 delivery also tended to have a small direct effect on the liver (NHGB= -3.0 ± 0.4 vs. -2.4 ± 0.4 mg/kg/min, during final 2 hours, not significant, p=0.30; PoGlu-GLP-1 and PoGlu-SAL) (Figure 4.6A). Collectively, therefore, our data suggest that physiological increases in GLP-1 can have a modest direct stimulatory effect on the liver.

In the present study, the groups that received an intraportal glucose infusion exhibited greater net hepatic glucose uptake (2.4 ± 0.4 and 3.0 ± 0.4 mg/kg/min, final 2 hours; PoGlu-SAL and PoGlu-GLP-1, respectively), than the groups that received only peripheral infusion of glucose (1.2 ± 0.1 and 1.6 ± 0.2 mg/kg/min; PeGlu-SAL and PeGlu-GLP-1, respectively, during final 2 hours). This increase in net hepatic glucose uptake in the presence of intraportal glucose delivery was probably attributable to both the greater (~30%) hepatic glucose load (239) and the portal signal per se (269).

In contrast to the results of our earlier studies, portal glucose infusion in the current study did not significantly decrease nonhepatic glucose uptake or increase insulin secretion (271; 272; 279). There was however, a trend toward a decrease in nonhepatic glucose uptake (3.8 ± 0.7 vs. 4.9 ± 1.0 mg/kg/min, final 2 hours; PoGlu-SAL and PeGlu-SAL) (Figure 4.6C, 4.6D) and higher hepatic portal vein insulin levels in the animals that received intraportal glucose compared to those that did not. There are two possible explanations for the lack of significant change in these parameters. First, the effect of intraportal glucose infusion on nonhepatic glucose uptake and plasma insulin levels may have been too small to detect given the limited power of this study. Second, in our earlier studies that demonstrated decreased nonhepatic glucose uptake and increased insulin secretion there were greater intraportal glucose infusion rates (5 and 10 mg/kg/min) (271;

272; 279) and greater peripheral glycemia (220 mg/dl) (272) than in the current study. Therefore, given the smaller portal glucose signal and the lower glucose level, it is not surprising that the changes in nonhepatic glucose uptake and insulin secretion did not reach statistical significance.

It has been well established that exogenously infused GLP-1 acts as an incretin in both healthy humans and those with Type 2 diabetes (40). As noted above, however, in Specific Aim I, as well as in the current study, there was no difference in arterial or portal plasma insulin levels in the presence or absence of GLP-1 infusion. This agrees with earlier data which showed that dogs which received a systemic infusion of glucose to simulate postprandial peripheral glucose levels showed no change in insulin levels when GLP-1 was infused peripherally to create a physiological increase in its level (49). The fact that a physiological elevation in GLP-1 did not result in changes in pancreatic hormone levels in the dog was discussed at length in Specific Aim I.

In conclusion, we have shown that a physiological elevation of plasma GLP-1 in the hepatic portal vein increases nonhepatic glucose uptake only when intraportal glucose delivery is also present. Furthermore, this effect is not related to differences in plasma insulin or glucagon levels. The data thus suggest that the GLP-1 secretion that occurs following feeding plays a role in limiting postprandial hyperglycemia by increasing liver and muscle glucose uptake even when the peptide does not augment the elevation in insulin or the decrease in plasma glucagon levels.

CHAPTER V

DURATION OF FAST INFLUENCES THE ACTIONS OF INTRAPORTALLY DELIVERED GLP-1

Specific Aim III-Introduction

As an incretin hormone, GLP-1 is most strongly associated with an insulin secretory effect; however, it has also been shown that the peptide has other gluco-regulatory actions, including increasing insulin mediated glucose uptake (48). These characteristics have made GLP-1 analogs and molecules which alter GLP-1 degradation promising therapies for type 2 diabetes, in that they target two contributing factors to the disease: inadequate insulin secretion and decreased insulin sensitivity.

In Specific Aim I, increased glucose and insulin, in combination with intraportal delivery of GLP-1 at rate which created a physiological increase in GLP-1 levels increased whole body glucose uptake in the 42 h fasted dog (268). This occurred without GLP-1 induced changes in the secretion of the pancreatic hormones (268). As shown in Specific Aim II, this effect on whole body glucose utilization only occurred under simulated feeding conditions when the plasma glucose level in the hepatic portal vein was elevated to levels greater than those in arterial blood (268; 280).

It has been shown in the human (281-283) that duration of fast is inversely correlated with whole body insulin sensitivity. In addition, it has been shown that a fast of shorter duration is associated with increased sensitivity of the β-cell to glucose, resulting in significantly increased glucose-dependent insulin secretion (283; 284). In the current study, we explored the possibility that, in a state of augmented β-cell and insulin

sensitivity, the magnitude of the effects of GLP-1 on glucose homeostasis would be increased.

Specifically, our aim was to determine if a physiologic increase in GLP-1 (47), caused by intraportal GLP-1 infusion, would result in a greater increase in whole body glucose uptake and/or greater insulin secretion, in dogs that were fasted for 18 h rather than for 42 h, by which time insulin sensitivity and β-cell responsiveness are markedly reduced.

Results

Plasma glucose levels. Plasma glucose levels in the artery (161 ± 3 and 162 ± 2 mg/dl) and hepatic portal vein (181 ± 2 and 184 ± 3 mg/dl) increased similarly in response to the combination of portal and peripheral glucose infusion in the SAL and GLP-1 groups, respectively (Figure 5.1A).

Plasma GLP-1 levels. There was no difference between groups in basal GLP-1 levels in either arterial or hepatic portal vein blood (Figure 5.1B), nor did the levels change in response to saline infusion. On the other hand, they rose in both the artery (to 30.3 ± 2.2 pM) and hepatic portal vein (to 50.3 ± 5.3 pM) in response to intraportal GLP-1 infusion (Figure 5.1B).

Glucose infusion rate. There was no difference in the total glucose infusion rates required to maintain the glucose clamp between the two groups (10.6 ± 1.3 vs. 9.7 ± 1.5 mg/kg/min, average over final 2 hours; SAL vs. GLP-1) (Figure 5.2).

Plasma insulin and glucagon levels. The arterial and hepatic portal vein plasma insulin levels were similar between groups during the basal period (Figure 5.3A). Both groups

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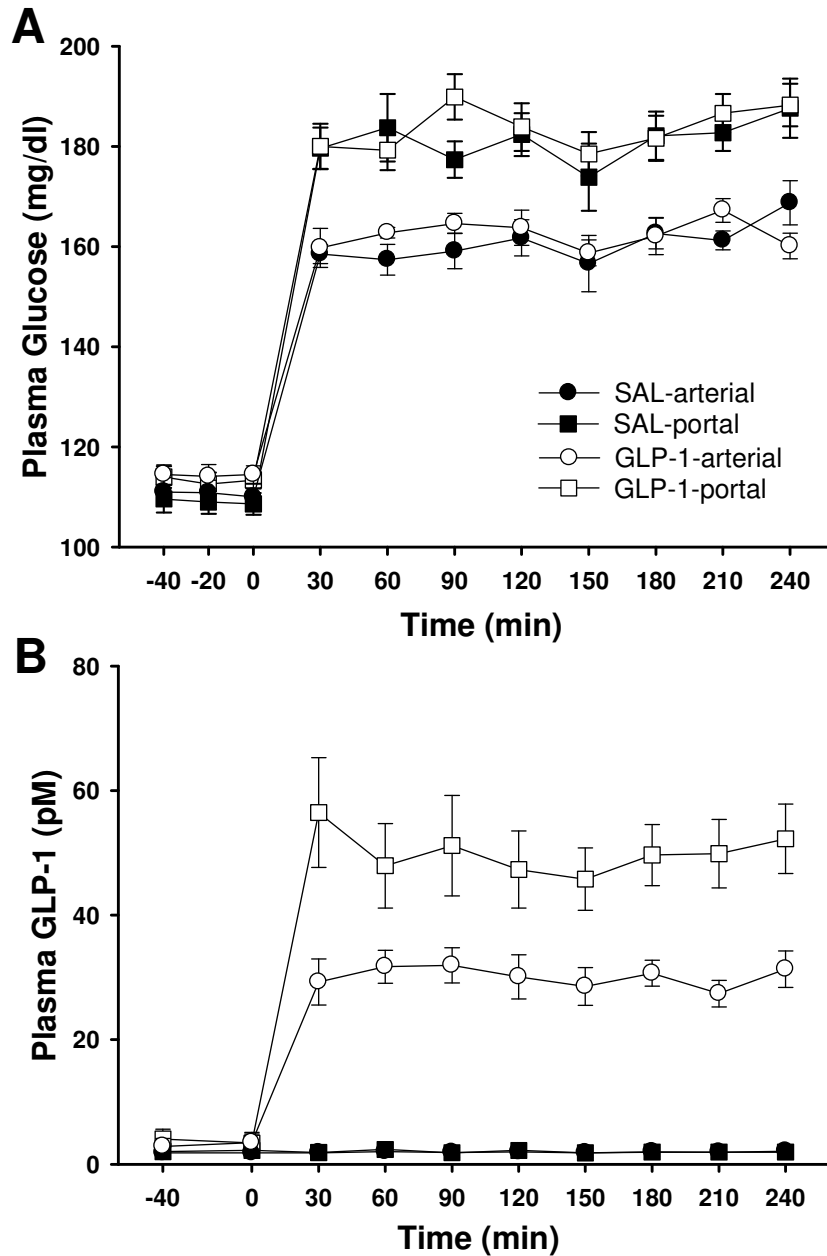
Figure 5.1 Plasma glucose and GLP-1 levels in 18-h fasted conscious dogs. *A*: Arterial and portal plasma glucose levels for dogs that received either intraportal GLP-1 or saline (SAL). Levels were basal initially (-40 to 0 min), but both arterial and portal levels increased significantly ($p < 0.05$) during the experimental period (0 to 240 min) in response to the glucose clamp. There were no significant differences between groups in either the basal or experimental period. B: Arterial and portal plasma GLP-1 levels for dogs that received either intraportal GLP-1 or saline. In the animals that received the GLP-1 infusion, GLP-1 levels were basal initially (-40 to 0 min), but both arterial and portal levels increased significantly ($p < 0.05$) during the experimental period (0 to 240 min). Levels remained unchanged in SAL. Data are expressed as mean ± SE.
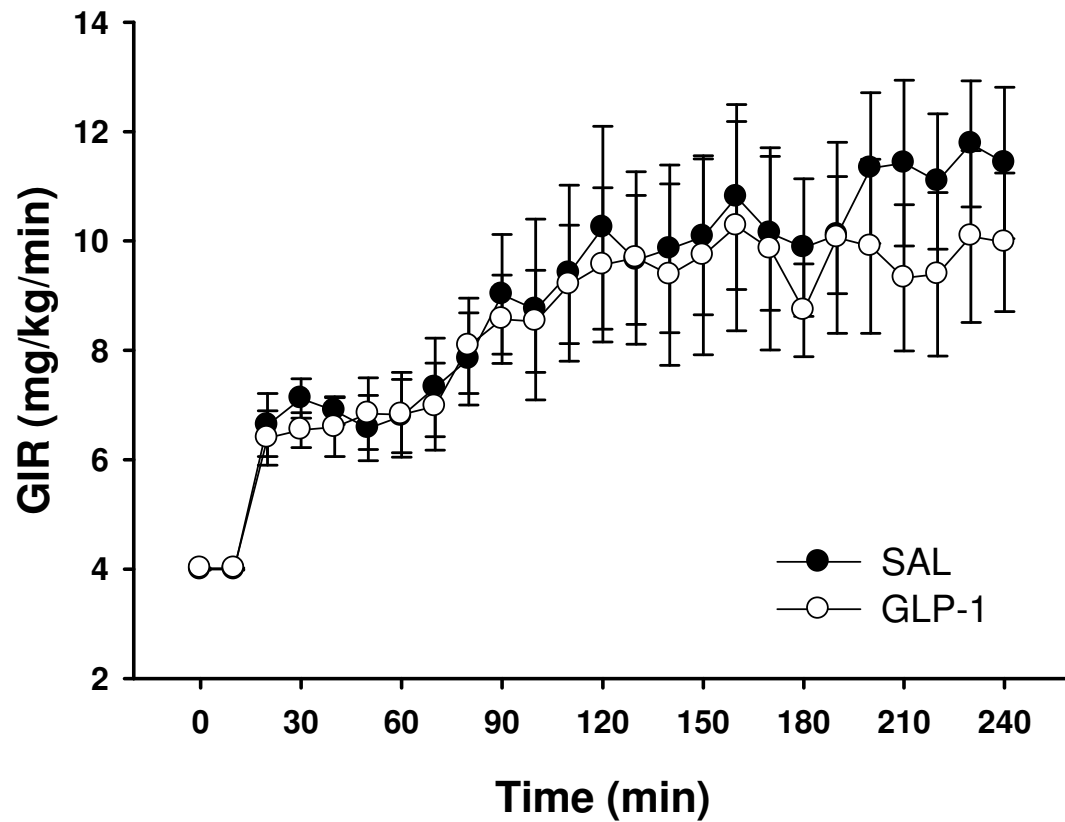
Figure 5.2 Total glucose infusion rate (GIR) during the infusion of intraportal GLP-1 or saline (SAL) into the hepatic portal vein (0 to 240 min). There was no statistical difference between groups. Data are expressed as mean ± SE for each time point.
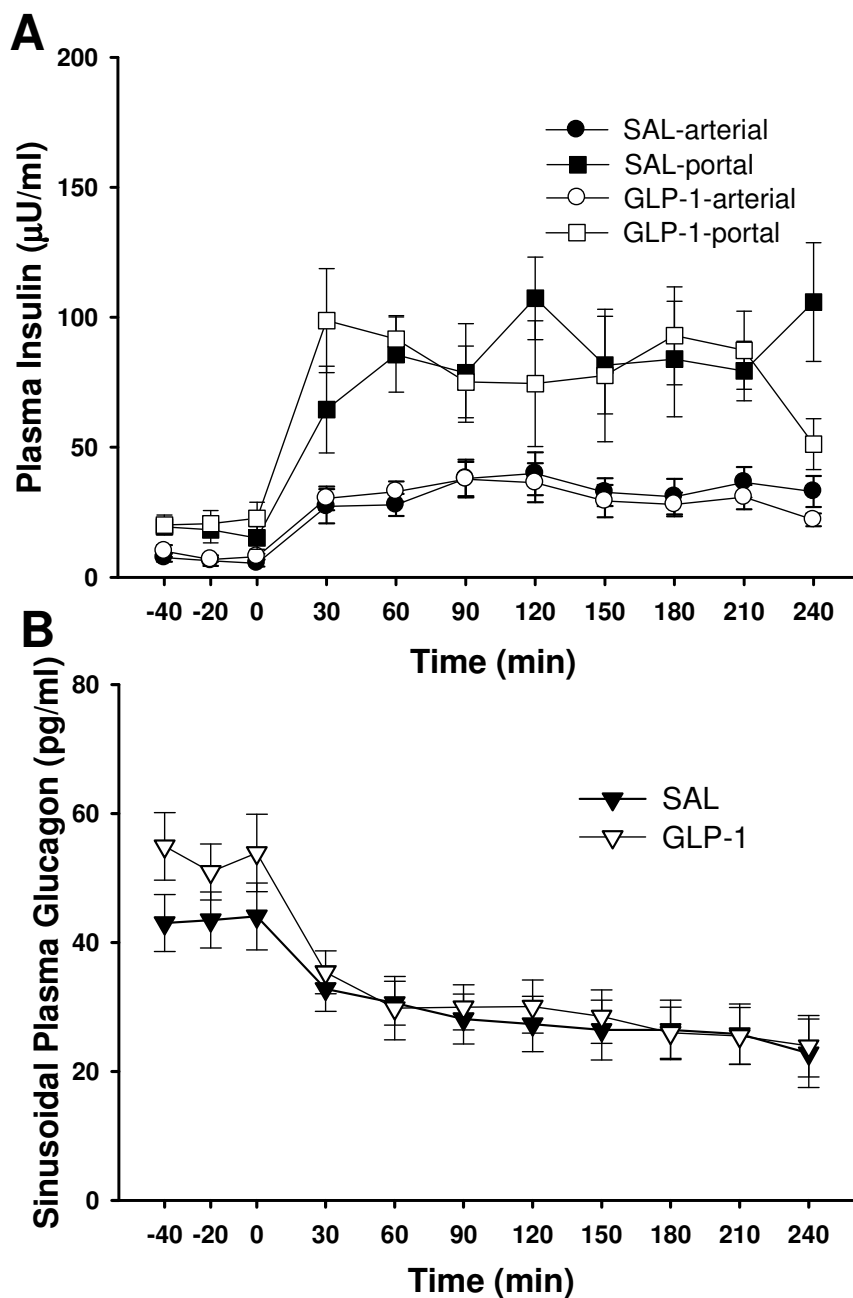
Figure 5.3 Plasma insulin and glucagon levels. *A*: Arterial and portal plasma insulin levels for dogs that received either intraportal GLP-1 or saline (SAL). Levels were basal initially (-40 to 0 min), but both arterial and portal levels increased significantly (p < 0.05) during the experimental period (0 to 240 min) in response to the glucose clamp. *B*: Sinusoidal plasma glucagon levels for animals that received either intraportal GLP-1 or saline. There was a significant decrease sinusoidal glucagon levels in both groups during the experimental period when compared to respective basal period values (p < 0.05). Data are expressed as mean ± SE.

exhibited similar increases in response to hyperglycemia (to 33 ± 4 and 31 ± 3 μU/ml in arterial plasma, and to 86 ± 8 and 81 ± 11 μU/ml in hepatic portal vein plasma in the SAL and GLP-1 groups, respectively, averaged over the entire experimental period) (Figure 5.3A). The sinusoidal plasma glucagon levels were not different between the two groups during the basal period (Figure 5.3B) and decreased in a similar manner (to 28 ± 4 and 29 ± 4 pg/ml in SAL and GLP-1, respectively, when averaged over the entire experimental period).

Hepatic blood flow, HGL, NHGB and non-HGU. Hepatic arterial blood flows during the basal period and the experimental period were similar between groups, as were the hepatic portal vein blood flows. In the basal state, net hepatic glucose output (Figure 5.4) was similar between groups (2.2 ± 0.2 and 1.6 ± 0.3 mg/kg/min; SAL and GLP-1, respectively). In response to the hyperglycemic clamp, the liver switched to net glucose uptake, with no significant difference between groups (NHGB = -3.6 ± 0.5 and -3.6 ± 0.5 mg/kg/min; SAL and GLP-1, respectively, during final 2 hours) (Figure 5.4A). There was no effect of GLP-1 on non-HGU (7.1 ± 1.5 and 6.2 ± 1.4 mg/kg/min, final 2 hours; SAL and GLP-1) (Figure 5.4B).

Arterial plasma free fatty acids. Arterial plasma FFA decreased in response to the hyperglycemia and hyperinsulinemia (from 817 ± 160 and 794 ± 114 to 147 ± 26 and 178 ± 31 μmol/l; SAL and GLP-1, respectively).
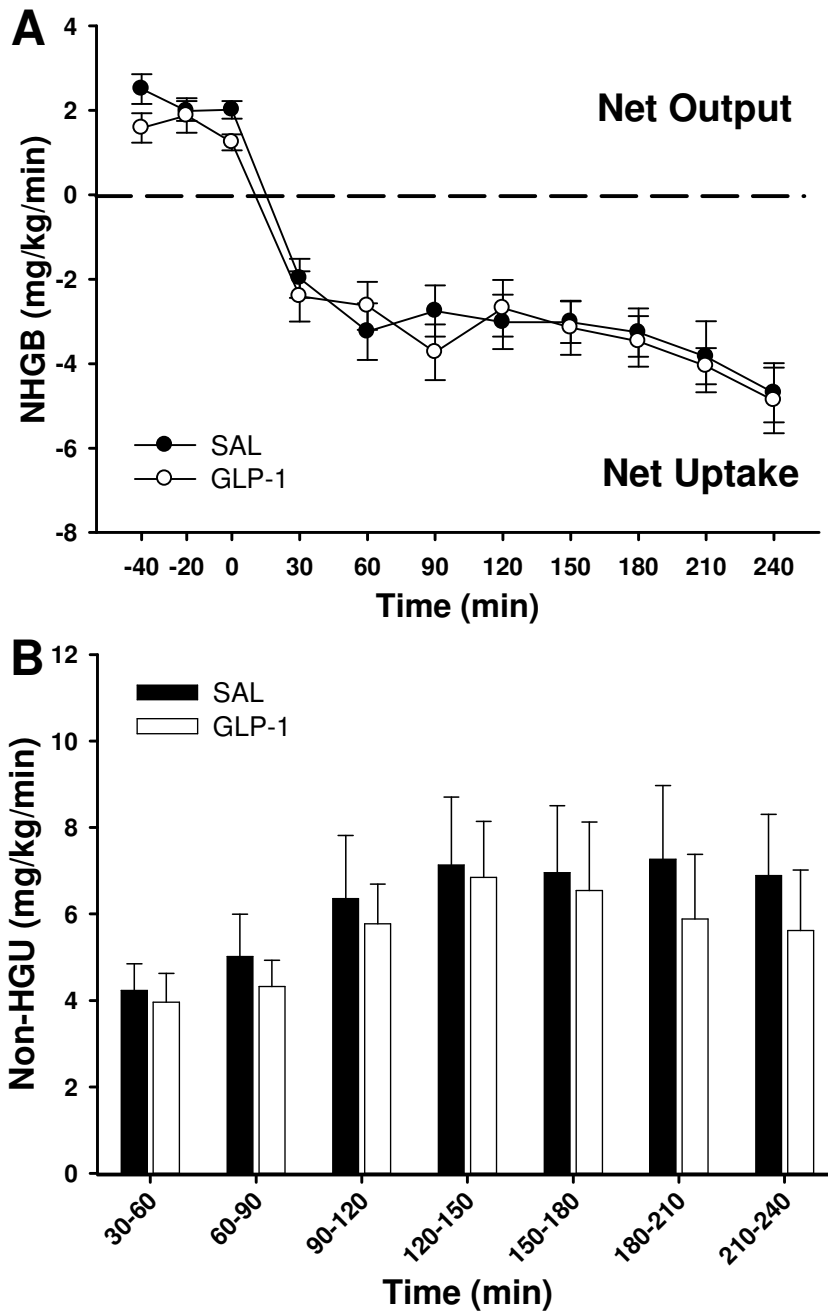
Figure 5.4 Glucose production and utilization. *A*: NHGB in animals that received either intraportal GLP-1 or saline (SAL). Rates in each group were significantly decreased (p < 0.05) during the experimental period when compared to their respective basal period values. There was no significant difference between groups. Data are expressed as mean ± SE. *B*: Non-HGU during the infusion of intraportal saline or GLP-1 during the experimental period (30 to 240 min). There was no significant difference between groups. Data are the average of values over 30 min segments and are expressed as mean ± SE.

Discussion

In the current study, conscious dogs were fasted for 18 hours, and then underwent a hyperglycemic clamp in the presence of either GLP-1 or saline given intraportally. The glucose and GLP-1 infusion rates were such that they brought about increases in plasma glucose and GLP-1 that resemble those seen after an oral glucose tolerance test (47). Glucose was infused through both the hepatic portal vein (4.0 mg/kg/min) and a peripheral vein (as needed) to clamp the arterial plasma glucose level at 160 mg/dl; however, pancreatic hormones were not clamped. In Specific Aim I, when this protocol was used in 42 hour fasted animals there was a significant increase in nonhepatic glucose uptake (Table 6.1) in response to GLP-1 infusion (268). This response occurred without any effect of GLP-1 on the pancreatic hormone levels (268). The question thus arises as to whether the long fast duration had in some way limited GLP-1's effects. The purpose of the current study, therefore, was to repeat our earlier experiment in animals that had only been fasted briefly and, as a consequence, had increased whole body insulin sensitivity and greater β-cell responsiveness (281-284). We hypothesized that under these conditions, GLP-1 infusion into the portal vein would cause greater nonhepatic glucose uptake than that seen in the previous study (268), in part at least because of an increase in insulin secretion.

In contrast to Specific Aim I, however, in the current study there was no difference in the glucose infusion rate required to maintain the glucose clamp when GLP-1 was infused intraportally as opposed to when saline was given via the portal vein (9.7 ± 1.5 vs. 10.6 ± 1.3 mg/kg/min, average over final 2 hours; GLP-1 vs. SAL) (Figure 6.2). In addition, as was the case Specific Aims I & II, there was no effect of GLP-1 on insulin or

TABLE 5.1

Comparison of glucose utilization parameters in 18 h fasted animals and 42 h fasted animals from Specific Aim I.

	18 h fast		42 h fast	
	Saline	GLP-1	Saline	GLP-1
Experimental Period Arterial Plasma Insulin (µU/ml)	33 ± 4	31 ± 3	*24 ± 2	*22 ± 3
Last 2 hr Arterial Plasma Insulin (µU/ml)	35 ± 5	29 ± 3	*23 ± 2	23 ± 3
Last 2 hr Hepatic Sinusoidal Plasma Insulin (µU/ml)	81 ± 10	67 ± 12	68 ± 5	60 ± 8
Last 2 hr Glucose Infusion Rate (mg/kg/min)	10.6 ± 1.3	9.7 ± 1.5	*6.0 ± 0.5	†8.5 ± 0.7
Last 2 hr Glucose Infusion Rate/				
** Arterial Plasma Insulin Ratio (mg/kg/min / µU/ml)**	0.35 ± 0.08	0.35 ± 0.05	0.27 ± 0.03	†0.43 ± 0.06
Last 2 hr Non-HGU (mg/kg/min)	7.1 ± 1.5	6.2 ± 1.4	3.8 ± 0.7	†5.5 ± 0.8
Last 2 hr Non-HGU/				
** Arterial Plasma Insulin Ratio (mg/kg/min / µU/ml)**	0.23 ± 0.08	0.21 ± 0.04	0.17 ± 0.03	0.29 ± 0.07
Last 2 hr Net Hepatic Glucose Uptake (mg/kg/min)	3.6 ± 0.5	3.6 ± 0.5	*2.4 ± 0.4	3.0 ± 0.4
Last 2 hr Net Hepatic Glucose Uptake/				
** Hepatic Sinusoidal Plasma Insulin (mg/kg/min / µU/ml)**	0.047 ± 0.009	0.066 ± 0.014	0.037 ± 0.008	0.059 ± 0.014

Mean ± SE; *$P < 0.05$ vs.18 h; †$P < 0.05$ vs. saline.

glucagon levels in plasma (268; 280) (Figure 6.3). Thus intraportal GLP-1 infusion did

not have a greater effect on nonhepatic glucose uptake in the 18 h fasted dog than it did in

animals fasted for 42 h (268), in fact, it had no effect whatsoever.

The total glucose infusion rate required to maintain arterial plasma glucose at 160

mg/dl in the 18 h fasted animals that received saline infusion in the current study was

significantly greater than the infusion rate required to maintain a similar clamp in 42 h

fasted dogs in Specific Aim I (10.6 ± 1.3 vs. 6.0 ± 0.5 mg/kg/min, average over the final

2 hours; 18 vs. 42 h fast, $p<0.01$) (268). This can mainly be attributed to significantly

greater arterial plasma insulin levels during the experimental period (33 ± 4 vs. 24 ± 2

μU/ml; 18 vs. 42-h fast, $p=0.02$) (268), which probably resulted from increased glucose

sensitivity at the β-cell (283). Insulin action, when expressed as a ratio of glucose uptake

to insulin level, tended to be greater in the 18 h fasted animals. Using these parameters,

insulin sensitivity increased in both hepatic (0.047 ± 0.009 vs. 0.037 ± 0.008 mg/kg/min /

μU/ml; 18 h saline vs. 42 h saline) and nonhepatic (0.23 ± 0.08 vs. 0.17 ± 0.03

mg/kg/min / μU/ml; 18-SAL vs. 42-SAL) (Table 6.1) tissues by approximately 30% after

the shorter fast. It has previously been shown that when glucose was delivered

intraduodenally, 42 h fasted dogs had significantly greater circulating plasma glucose

levels than 18 h fasted animals given the same glucose load; nevertheless, the 18 h fasted

dogs maintained the same rate of glucose uptake by the liver and hindlimb as 42 h fasted

dogs, even though the glucose loads (levels) to both of these tissues was significantly

reduced in the shorter fasted animals (285). This agrees with the increase in insulin

sensitivity caused by a decrease in fast duration observed in the current study. In addition,

despite the increased arterial glucose level in the 42 h fasted animals that received

intraduodenal glucose, the 18 h and 42 h fasted groups had statistically similar arterial plasma insulin levels (285), confirming increased β-cell glucose sensitivity in the shorter fasted dogs.

There was no significant difference in the glucose infusion rates required to clamp the arterial plasma glucose level at 160 mg/dl in response to the combination of increased GLP-1, glucose, and insulin (9.7 ± 1.5 vs. 8.5 ± 0.7 mg/kg/min, average over final 2 hours) in 18 vs. 42 h fasted dogs (268). This indicates that intraportal GLP-1 infusion ameliorated the impaired insulin sensitivity evident in a 42 h fasted dog, but was without effect when insulin sensitivity was normal.

The question thus arises as to how a physiological increase in the plasma GLP-1 level could restore glucose utilization in the 42 h fasted dog, in Specific Aim I, to a rate not significantly different from that observed in an 18 h fasted animals. The decreased insulin sensitivity associated with an extended fast or calorie restriction is thought to be due to impaired insulin signaling (286) which results from increased circulating FFA levels and increased fat oxidation (282; 287; 288). In addition, increased FFA levels have been shown to result in β-cell impairment (289). Since others have shown that GLP-1 infusion can decrease circulating FFA (86), one might postulate that the plasma FFA were decreased to a greater extent in the GLP-1 infused 42 h fasted dogs than in the saline infused animals. Indeed, this was the case. The dogs which received GLP-1 in Specific Aim I had a lower level of plasma FFA during the experimental period than the saline infused dogs (168 ± 14 vs. 362 ± 111μmol/l, 42 h GLP-1 vs. 42 h saline, respectively) (268). In fact, the arterial plasma FFA levels were decreased by GLP-1 in the 42 h fasted dogs to the levels observed during the experimental period in the 18 h

fasted dogs that received either GLP-1 (178 ± 31 μmol/l) or saline (147± 26 μmol/l).

Further analysis of the data, however, does not support this hypothesis. If one considers

the four saline infused animals (of a total eight) from Specific Aim I (42 h fasted dogs)

which had the lowest arterial plasma FFA levels, it becomes obvious that their arterial

plasma FFA levels fell to the level observed in the 42 h fasted GLP-1 infused animals

(168 ± 14 vs. 172 ± 32 μmol/l; 42 h GLP-1 vs. 42 h saline-low FFA) (268). The glucose

infusion rate in this subgroup (6.6 ± 0.6 mg/kg/min, average over final 2 hours) was still

very different from that in the GLP-1 group (8.5 ± 0.7 mg/kg/min, average over final 2

hours) (268). Thus although glucose infusion was greater by 1.2 mg/kg/min in the

controls which had lower FFA level, than the four saline infused 42 h fasted dogs with

higher FFA levels (5.4 ± 0.9 vs. 6.6 ± 0.6 mg/kg/min, average final 2 hr; 42 h saline-high

FFA vs. 42 h saline-low FFA) (268), this does not provide an explanation for GLP-1's

actions. It remains to be determined what aspect of fasting associated with insulin

resistance was mitigated by the physiological increase in GLP-1.

Differences in the effects of GLP-1 due to difference in fasting are not

unprecedented. While GLP-1 does inhibit neural signals that trigger small bowl motility

in fed rats, the amount of GLP-1 required for the same effect is significantly decreased in

the fasted state (213; 290). It has even been suggested that the effect of GLP-1 on gut

motility in the fasted and fed states actually are the result of two separate mechanisms,

either dependent or independent, respectively, of nitric oxide signaling (213). In

addition, it appears as though leptin, the circulating level of which decreases with fasting,

augments the effect of GLP-1 on food intake only in the fed state when leptin levels are

increased (291).

Absence of a change in pancreatic hormone levels in response to a physiological increase in GLP-1 levels in the dog is in agreement with Specific Aims I & II (268; 280) and that of others (237). This issue has been addressed in previous aims (268; 280); however, in brief, it appears as though in the dog a physiological increase of GLP-1 delivered via the hepatic portal vein does not have an incretin effect. Despite this finding, one cannot conclude that endogenously released GLP-1 does not have an incretin effect in the dog. Such an action could be initiated by a neural signal closer to the L cell, possibly via an interaction of GLP-1 at the local level with the afferent vagal nerve endings in the basolateral membrane of the entero-endocrine cells, much in the same manner as cholecystokinin (CCK) interacts at these nerve endings to initiate its gastric effects (292).

In the dog, glucose responsiveness of the β-cell is greater in 18 h than 42 h fasted animals, and there tends to be an increase in insulin sensitivity at liver and muscle. Together, these actions result in more efficient whole body glucose utilization. The results of the current study indicate that, in 18 h fasted dogs, a physiological increase in GLP-1 levels does not alter insulin secretion. Additionally, unlike the case with 42 h fasted dogs in Specific Aim I, GLP-1 did not increase nonhepatic glucose utilization in 18 h fasted dogs. This suggests that GLP-1 can improve nonhepatic glucose uptake without altering pancreatic hormone secretion, but only when the former is impaired, as in the long-fasted state.

CHAPTER VI

THE ROLE OF ENDONGENOUSLY RELEASED GLP-1 IN GLUCOSE REGULATION IN NORMAL AND INSULIN RESISTANT DOGS

Specific Aim IV-Introduction

Approximately half of all secreted GLP-1 is degraded between the site of secretion and the portal vasculature (58). It is possible that, upon secretion, GLP-1 acts in a paracrine manner by activating receptors in the vasculature close to the site of secretion, upstream from the hepatic portal vein. In the gut, vagal afferents terminate in the region of the basolateral membrane of entero-endocrine cells, an established mechanism by which cholecystokinin (CCK) regulates gastric emptying and food intake (292). It is therefore possible that GLP-1 acts in a similar manner.

In the previous aims, GLP-1 was infused into the hepatic portal vein to create a physiological increase in the levels of the peptide, so that levels in the hepatic portal vein were approximately twice those observed in the periphery, mimicking levels observed after an OGTT (47); however, intraportal infusion does not elevate GLP-1 in the vasculature surrounding the L cell, as would occur in response to endogenous GLP-1 secretion. Although GLP-1 is best known for its action at the β-cell, intraportal GLP-1 infusion did not alter pancreatic hormone levels in any of the previous protocols. It is possible that the contribution of GLP-1 to the incretin effect is mediated through its elevation in direct proximity to its site of secretion. Endogenously secreted GLP-1 may also have other effects on postprandial glucose disposition which were not observed in the previous protocols.

It has been established in the human that one effect of a physiological rise in GLP-1 after a meal is slowed gastric emptying (293). In agreement with that, circulating postprandial GLP-1 levels are inversely correlated to the rate of gastric emptying (214). In humans, when GLP-1 is infused during a meal, postprandial glycemia is decreased, and insulin levels are below those seen in the absence of GLP-1 (210). This indicates that increased insulin is not the cause of the decreased blood glucose level. When GLP-1 action is blocked in humans after an OGTT, peripheral plasma glucose levels are elevated, and this is accompanied by an appropriately elevated insulin level (294). Combined, these results indicate that a primary role of endogenously secreted GLP-1 in glucose regulation may be to slow gastric emptying (210), which inturn would limit the elevation of glycemia.

Specific Aim I established that intraportal GLP-1 delivery increased nonhepatic glucose uptake (268). In Specific Aim II, it was determined that this action was dependent on intraportal glucose delivery (280), essentially recreating arterial-hepatoportal gradients of both GLP-1 and glucose observed after a meal. Results from Specific Aim I and II (268; 280), from previous work done in our lab (47), and from the work of others (98), indicate that GLP-1 can have a direct effect on hepatic glucose uptake. Therefore, glucose uptake could be another way in which endogenously secreted GLP-1 decreases postprandial glucose levels.

Specific Aim IV was to determine if endogenously released GLP-1 has any impact on postprandial glucose disposal in the dog through its effects on the pancreas, gastric emptying, and/or glucose utilization. To execute this aim we studied healthy dogs and an additional subset of insulin resistant dogs, twice each. The animals received either

a peripheral saline or exendin (9-39) (Ex-9) infusion in random order. Ex-9 is an antagonist of the GLP-1R. During both studies the dogs received an orally delivered liquid meal consisting of carbohydrate, fat, and protein, to induce endogenous GLP-1 secretion. The meal was spiked with acetaminophen to quantify gastric emptying. Effects of endogenous GLP-1 secretion on pancreatic hormone secretion, glucose utilization, and gastric emptying were assessed.

Results

Plasma GLP-1 Levels. In the *normal dogs*, arterial plasma GLP-1 levels were at a minimum during the basal period (3.6 ± 1.8 and 5.6 ± 2.9 pM, saline and Ex-9, respectively) and during the infusion of saline or Ex-9 (3.5 ± 1.8 and 5.0 ± 2.8 pM, t=30 min) prior to the meal (Table 6.1). GLP-1 levels increased in response to the meal to 9.7 ± 2.6 pM (45 to 200 min) with saline infusion and 16.7 ± 4.4 pM with Ex-9 infusion (Table 6.1). In the *insulin resistant dogs*, deep venous plasma GLP-1 levels were minimal during the basal period (0.3 ± 0.3 and 0.1 ± 0.1 pM, saline and Ex-9, respectively) and during the infusion of saline or Ex-9 (0.2 ± 0.3 and 0.2± 0.2 pM, t=30 min) prior to the meal (Table 6.1). After meal administration (45 to 200 min) levels reached 1.5 ± 1.5 pM with saline infusion and 2.3 ± 2.0 pM with Ex-9 infusion (Table 6.1).

Plasma Acetaminophen Levels. To assess gastric emptying circulating plasma acetaminophen levels were measured. After administration of the meal to *normal dogs*, arterial plasma acetaminophen levels were significantly lower (p=0.04) when saline was infused (6.7 ± 1.1 µg/ml from 45 to 200 min) than when Ex-9 was infused (8.2 ± 0.9 µg/ml (Figure 6.1A). The area under the curve for the increase from baseline (Δ AUC)

TABLE 6.1
Levels observed during basal (-20 to 0 min) and experimental period, both prior (0 to 30 min) and after (30 to 320 min) meal administration. Data are expressed as mean ± SE.
*=P<0.05 compared to saline.

	Basal Period	Experimental Period								
		30	45	60	80	110	140	200	260	320
Plasma GLP-1 Levels (pM)										
Normal Dogs (n=8) - Arterial Plasma GLP-1										
Saline	3.6 ± 1.8	3.5 ± 1.8	10.6 ± 5.4	10.3 ± 3.4	7.8 ± 3.0	8.5 ± 2.9	8.4 ± 2.4	13.6 ± 3.3	15.1 ± 4.9	9.2 ± 3.7
Exendin (9-39)	5.6 ± 2.9	5.0 ± 2.8	8.3 ± 3.6	15.6 ± 8.5	14.1 ± 3.6	15.1 ± 4.5	16.6 ± 4.9	24.8 ± 8.1	25.1 ± 12.4	14.7 ± 6.5
Insulin Resistant Dogs (n=3) - Deep Venous Plasma GLP-1										
Saline	0.3 ± 0.3	0.2 ± 0.3	0.4 ± 0.5	0.8 ± 0.9	2.2 ± 2.5	1.4 ± 1.6	1.3 ± 1.6	2.0 ± 1.2	3.4 ± 4.2	1.7 ± 2.1
Exendin (9-39)	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	0.6 ± 0.4	4.1 ± 3.8	3.2 ± 2.0	2.4 ± 2.6	1.5 ± 1.1	0.4 ± 0.3	0.1 ± 0.1
Plasma C-Peptide Levels (ng/ml)										
Normal Dogs (n=8) - Arterial Plasma C-Peptide										
Saline	0.37 ± 0.07	0.31 ± 0.07	1.03 ± 0.40	1.33 ± 0.35	1.62 ± 0.32	0.93 ± 0.28	1.49 ± 0.44	1.45 ± 0.20	1.31 ± 0.19	0.97 ± 0.22
Exendin (9-39)	0.33 ± 0.10	0.36 ± 0.07	1.67 ± 0.63	1.88 ± 0.34	1.83 ± 0.26	1.66 ± 0.21	1.62 ± 0.19	1.45 ± 0.14	0.94 ± 0.26	0.83 ± 0.25
Insulin Resistant Dogs (n=3) - Deep Venous Plasma C-Peptide										
Saline	0.34 ± 0.08	0.50 ± 0.11	0.49 ± 0.18	0.84 ± 0.30	1.70 ± 0.54	1.84 ± 0.43	1.99 ± 0.48	1.99 ± 0.29	1.34 ± 0.39	1.21 ± 0.45
Exendin (9-39)	0.40 ± 0.08	0.39 ± 0.12	0.58 ± 0.09	0.87 ± 0.51	1.90 ± 0.84	*2.79 ± 0.14	2.50 ± 0.48	2.47 ± 0.25	1.22 ± 0.41	0.94 ± 0.23
Plasma Glucagon Levels (pg/ml)										
Normal Dogs (n=8) - Arterial Plasma Glucagon										
Saline	47 ± 5	44 ± 3	50 ± 3	43 ± 5	46 ± 6	42 ± 4	44 ± 5	44 ± 6	45 ± 9	44 ± 7
Exendin (9-39)	41 ± 7	40 ± 6	43 ± 8	40 ± 9	45 ± 9	42 ± 8	49 ± 9	52 ± 11	51 ± 10	49 ± 9
Insulin Resistant Dogs (n=3) - Deep Venous Plasma Glucagon										
Saline	43 ± 6	43 ± 10	60 ± 16	60 ± 9	61 ± 9	51 ± 6	51 ± 6	48 ± 3	44 ± 3	44 ± 6
Exendin (9-39)	42 ± 4	41 ± 5	51 ± 9	54 ± 12	62 ± 19	51 ± 8	51 ± 11	47 ± 6	36 ± 9	46 ± 8
Plasma Free-Fatty Acid Levels (μmol/l)										
Normal Dogs (n=8) - Arterial Plasma Free-Fatty Acids										
Saline	884 ± 143	912 ± 117	684 ± 153	408 ± 171	240 ± 95	217 ± 82	206 ± 76	142 ± 44	93 ± 29	138 ± 29
Exendin (9-39)	773 ± 106	770 ± 105	712 ± 143	422 ± 188	225 ± 89	146 ± 52	120 ± 36	158 ± 97	159 ± 74	168 ± 63
Insulin Resistant Dogs (n=3) - Deep Venous Plasma Free-Fatty Acids										
Saline	731 ± 90	738 ± 36	680 ± 75	577 ± 22	356 ± 132	180 ± 122	96 ± 58	51 ± 29	77 ± 12	199 ± 105
Exendin (9-39)	842 ± 81	758 ± 74	935 ± 193	734 ± 156	441 ± 179	176 ± 62	100 ± 51	70 ± 32	81 ± 33	301 ± 71
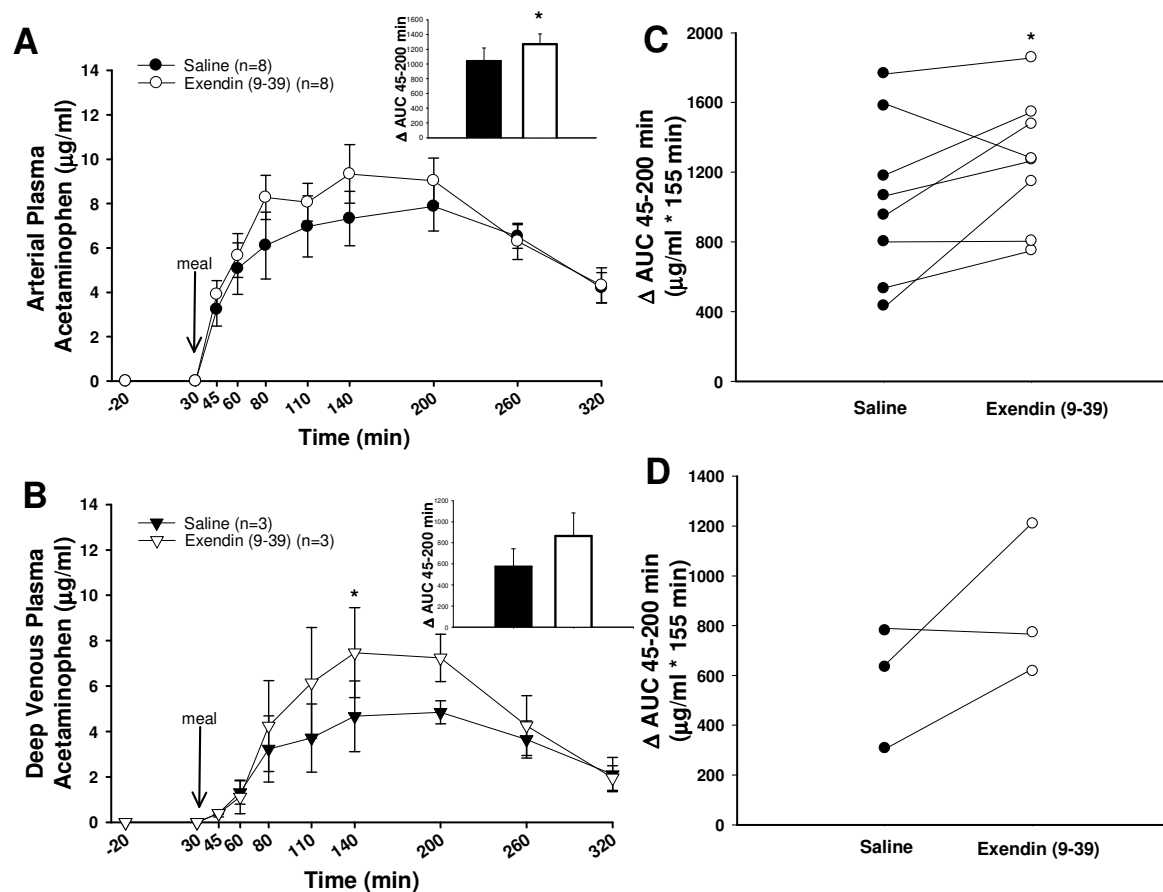
Figure 6.1 Plasma acetaminophen levels and Δ AUC. *A*: Arterial plasma acetaminophen levels for normal dogs after meal and Δ AUC from 45 to 200 min (inset). Data are mean ± SE. *: $P < 0.05$ compared to saline infusion. *B*: Deep venous plasma acetaminophen levels for insulin resistant dogs after meal and Δ AUC from 45 to 200 min (inset). Data are mean ± SE. *: $P < 0.05$ compared to saline infusion. *C*: Individual arterial plasma acetaminophen Δ AUC from 45 to 200 min for normal dogs. *: $P < 0.05$ compared to saline infusion. *D*: Individual deep venous plasma acetaminophen Δ AUC from 45 to 200 min for insulin resistant dogs.

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from 45 to 200 min was also significantly lower (p=0.04) with saline infusion (1041 ±

177 μg/ml*155 min) versus Ex-9 infusion (1268 ± 140 μg/ml*155 min) (Figure 6.1A,

inset, Figure 6.1C). This represents an 18 ± 10% decrease in the Δ AUC when GLP-1

was able to interact with its receptor. After administration of the meal to *insulin resistant*

dogs, deep venous plasma acetaminophen levels averaged 3.7 ± 1.1 μg/ml with saline

infusion (45 to 200 min; Figure 6.1B). With Ex-9 infusion, deep venous plasma

acetaminophen levels averaged 5.6 ± 1.4 μg/ml (45 to 200 min; Figure 6.3B). Δ AUC

from 45 to 200 min was 574 ± 171 μg/ml*155 min with saline infusion, which was 32 ±

20% lower than when Ex-9 was infused (866 ± 217*155 min) (Figure 6.1B, inset, Figure

6.1D).

Net Gut and Splanchnic Glucose Balance. We next determined the effect of gastric

emptying on net gut glucose absorption and net splanchnic glucose balance. Total hepatic

blood flow was nearly identical between the two protocols in *normal dogs* prior to meal

administration (Table 6.2). After administration of the meal, hepatic portal vein blood

flow increased by ~ 30% , regardless of treatment (Table 6.2). Net gut glucose output

after the meal (45 to 200 min) was 7.6 ± 1.1 mg/kg/min with saline infusion and 11.2 ±

2.2 mg/kg/min with Ex-9 infusion (Figure 6.2A). Net splanchnic glucose output after the

meal (45 to 200 min) was 4.5 ± 1.0 mg/kg/min with saline infusion and 7.8 ± 2.0

mg/kg/min with Ex-9 infusion (Figure 6.2B). These parameters were not measured in the

insulin resistant dogs.

Plasma Glucose. In the *normal dogs*, the basal arterial plasma glucose levels were 119 ±

2 and 118 ± 2 mg/dl prior to saline or Ex-9 infusion, respectively (Figure 6.3A). Saline

or Ex-9 were started at t=0 min and arterial plasma levels did not change from basal (118

TABLE 6.2

Blood flow in normal dogs during basal (-20 to 0 min) and experimental period, both prior (0 to 30 min) and after (30 to 320 min) meal administration. Data are expressed as mean ± SE.

	Basal Period	30	45	60	80	110	140	200	260	320
Hepatic Artery Blood Flow (ml/kg/min, n=8)										
Saline	7.2 ± 0.6	6.6 ± 0.6	7.8 ± 0.8	7.3 ± 0.8	7.2 ± 0.8	7.4 ± 1.0	7.3 ± 0.7	7.0 ± 0.4	6.9 ± 0.5	6.7 ± 0.4
Exendin (9-39)	6.1 ± 0.6	5.8 ± 0.6	7.1 ± 0.9	6.2 ± 0.9	6.3 ± 0.7	5.8 ± 0.5	6.1 ± 0.7	5.5 ± 0.4	5.5 ± 0.4	5.5 ± 0.4
Hepatic Portal Vein Blood Flow (ml/kg/min, n=8)										
Saline	29.2 ± 2.8	32.2 ± 2.8	34.0 ± 2.3	36.0 ± 2.1	35.9 ± 2.2	33.6 ± 2.5	32.7 ± 2.2	34.4 ± 2.3	35.6 ± 2.5	33.0 ± 2.3
Exendin (9-39)	28.2 ± 2.1	28.9 ± 2.0	36.3 ± 1.7	38.2 ± 2.1	38.6 ± 2.4	37.1 ± 3.3	38.7 ± 2.7	37.8 ± 2.5	37.0 ± 1.8	34.0 ± 2.1
Total Blood Flow (ml/kg/min, n=8)										
Saline	36.4 ± 3.2	38.8 ± 3.0	41.8 ± 2.6	43.3 ± 2.0	43.1 ± 1.9	41.0 ± 2.5	40.0 ± 1.9	41.5 ± 2.3	42.4 ± 2.5	39.7 ± 2.4
Exendin (9-39)	34.4 ± 2.4	34.7 ± 2.3	43.4 ± 2.2	44.4 ± 2.3	44.9 ± 2.6	42.9 ± 3.3	44.8 ± 2.5	43.3 ± 2.4	42.5 ± 1.9	39.5 ± 2.4
Hindlimb Blood Flow (ml/min, n=3)										
Saline	101 ± 11	120 ± 20	105 ± 11	102 ± 17	104 ± 7	114 ± 18	122 ± 15	100 ± 10	112 ± 22	147 ± 4
Exendin (9-39)	119 ± 6	132 ± 8	114 ± 12	138 ± 10	146 ± 16	128 ± 15	110 ± 8	169 ± 38	152 ± 24	202 ± 29
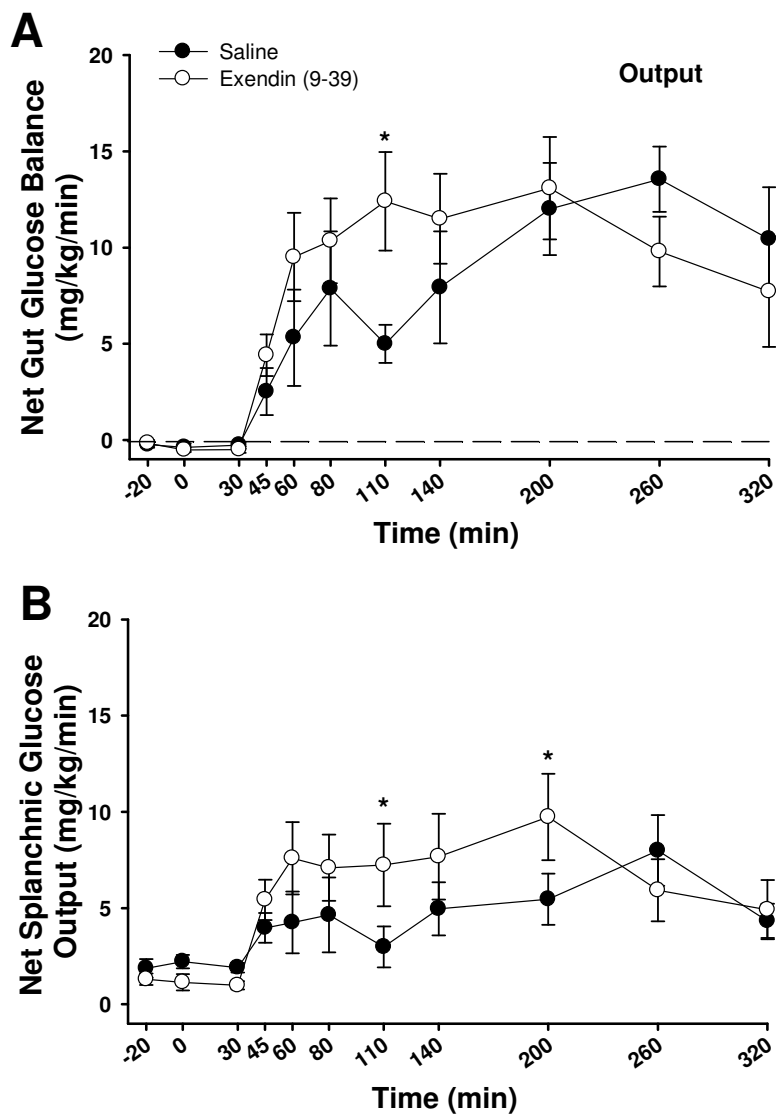
Figure 6.2 Net gut and splanchnic glucose balance during the basal period (-20 to 0 min), experimental period prior to meal (0 to 30 min), and experimental period post meal (30 to 320 min). *A*: Net gut glucose balance in normal dogs. *B*: Net splanchnic glucose balance in normal dogs. Data are mean ± SE. *: P < 0.05 compared to saline infusion.
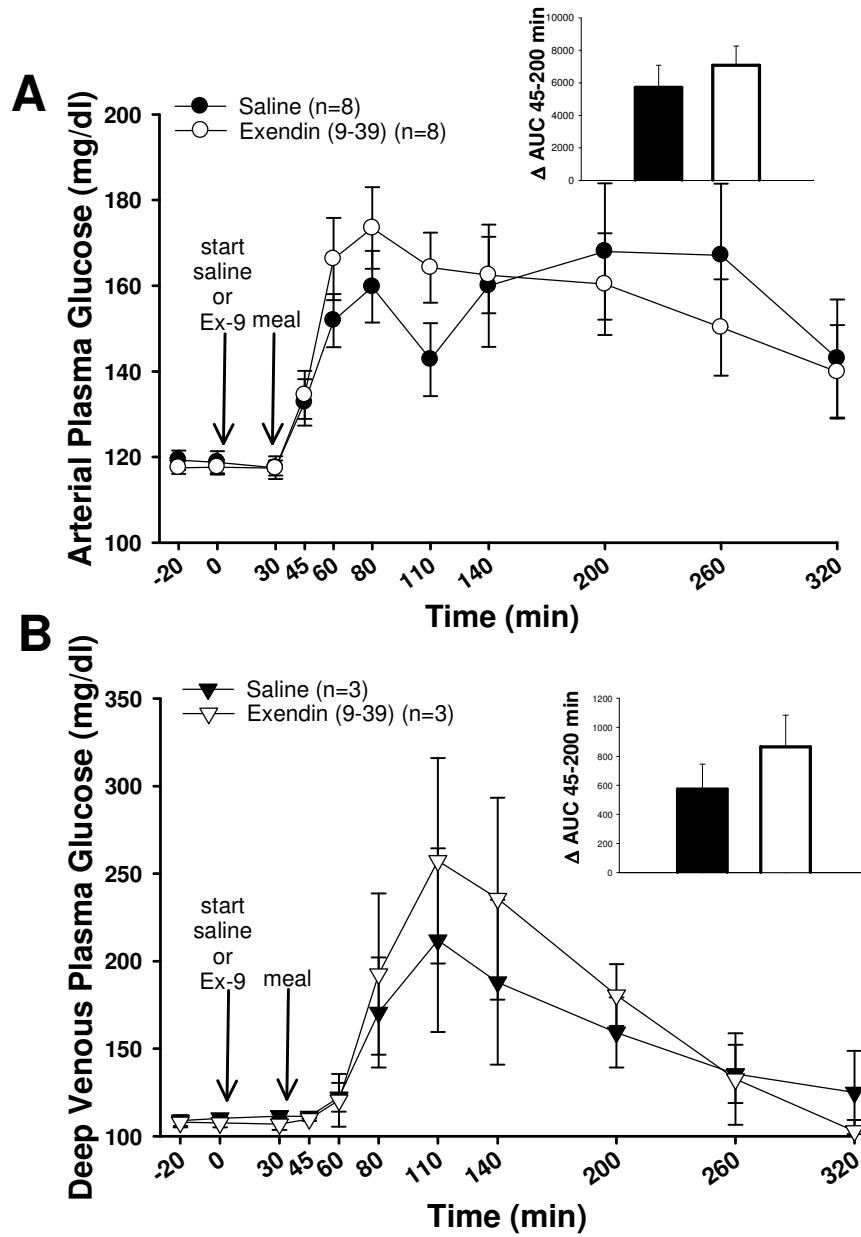
Figure 6.3 Plasma glucose levels during the basal period (-20 to 0 min), experimental period prior to meal (0 to 30 min), and experimental period post meal (30 to 320 min). *A*: Arterial plasma glucose levels for normal dogs and Δ AUC from 45 to 200 min (inset). *B*: Deep venous plasma glucose levels for insulin resistant dogs and Δ AUC from 45 to 200 min (inset). Data are mean ± SE.

± 3 and 117 ± 2 mg/dl, t=30 min). After administration of the meal, the arterial plasma

glucose levels averaged 156 ± 8 mg/dl during saline infusion, and 163 ± 7 mg/dl during

Ex-9 infusion (Figure 6.3A, 45 to 200 min). Δ AUC from 45 to 200 min was 5725 ±

1347 mg/dl*155 min and 7085 ± 1176 mg/dl*155 min, in the presence of saline and Ex-

9, respectively (Figure 6.3A, inset). This represents a 19 ± 12% decrease in the Δ AUC

when GLP-1 was able to interact with its receptor. In the *insulin resistant dogs*, deep

venous plasma glucose levels during the basal period (-20 to 0 min) were 110 ± 2 and

108 ± 3 mg/dl prior to saline or Ex-9 infusion, respectively. Plasma glucose did not

change in response to saline or Ex-9 infusion (112 ± 1 and 107 ± 3 mg/dl, t=30 min).

After the meal was administered the deep venous plasma glucose levels averaged 173 ±

33 mg/dl (45 to 200 min) during saline infusion and 203 ± 39 mg/dl with Ex-9 infusion

(Figure 6.3B). Δ AUC from 45 to 200 min was 9741 ± 4880 mg/dl*155 min with saline

and 14827 ± 5654 mg/dl*155 with Ex-9 infusion (p=0.06). This represents a 37 ± 12%

decrease when GLP-1 interacted with its receptor (Figure 6.1B, inset).

Plasma Insulin Levels. In the *normal dogs*, arterial plasma insulin levels during the basal

period (9 ± 2 and 8 ± 2 μU/ml, saline and Ex-9, respectively) were similar between the

two groups, and did not change during the infusion of saline or Ex-9 (9 ± 1 and 8 ± 2

μU/ml, t=30 min) prior to the meal (Figure 6.4A). After the meal was administered (45

to 200 min), the circulating insulin levels increased in response to hyperglycemia with

both saline (49 ± 9 μU/ml) and Ex-9 infusion (55 ± 7 μU/ml) (Figure 6.4A). The ratio of

arterial plasma insulin to arterial plasma glucose, prior to the meal, averaged 0.07 ± 0.01

and 0.07 ± 0.01 μU/mg (Figure 6.4C). After the meal (45 to 200 min), the average ratio

was 0.31 ± 0.07 and 0.34 ± 0.05 μU/mg with saline and Ex-9 infusion, respectively
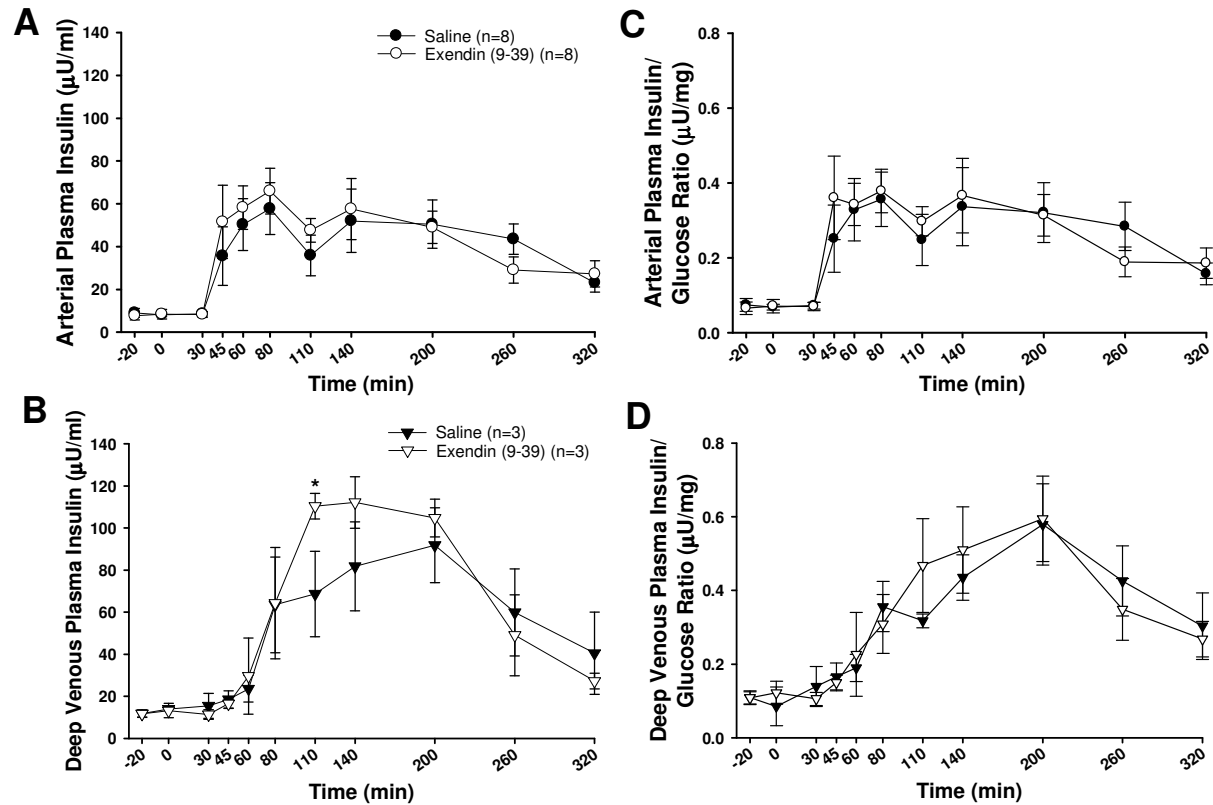
Figure 6.4 Plasma insulin levels and ratio of insulin levels to plasma glucose levels during the basal period (-20 to 0 min), experimental period prior to meal (0 to 30 min), and experimental period post meal (30 to 320 min). *A*: Arterial plasma insulin levels from normal dogs. *B*: Deep venous plasma insulin levels from insulin resistant dogs. *C*: Ratio of arterial plasma insulin to arterial plasma glucose levels from normal dogs. *D*: Ratio of deep venous insulin to deep venous plasma glucose levels from insulin resistant dogs. Data are mean ± SE. *: $P < 0.05$ compared to saline infusion.

(Figure 6.4C). In the *insulin resistant dogs*, deep venous plasma insulin levels during the

basal period (12 ± 1 and 12 ± 3 µU/ml, saline and Ex-9, respectively) were identical

between the two studies, and the levels did not change significantly during the infusion of

saline or Ex-9 (16 ± 6 and 11 ± 2 µU/ml, t=30 min) prior to the meal (Figure 6.4B).

After the meal was administered (45 to 200 min) the circulating insulin levels increased

in response to hyperglycemia during saline (69 ± 14 µU/ml) and Ex-9 infusion

(89 ± 6 µU/ml, p=0.05) infusion (Figure 6.4B). The ratio of arterial plasma insulin to

arterial plasma glucose prior to the meal averaged 0.11 ± 0.04 and 0.11 ± 0.01 µU/mg,

while the ratios after administration of the meal (45 to 200 min) were 0.39 ± 0.01 and

0.44 ± 0.06 µU/mg with saline and Ex-9 infusion, respectively (Figure 6.4D).

Plasma C-peptide Levels. In the *normal dogs*, arterial plasma C-peptide levels were

similar between treatments during the basal period (0.37 ± 0.07 and 0.33 ± 0.10 ng/ml,

saline and Ex-9, respectively), with no change during the infusion of saline or Ex-9 (0.31

± 0.07 and 0.36 ± 0.07 ng/ml, t=30 min) prior to the meal (Table 6.1). After the meal (45

to 200 min), C-peptide levels were 1.35 ± 0.26 ng/ml with saline infusion and 1.66 ± 0.15

ng/ml with Ex-9 infusion (Table 6.1). In the *insulin resistant dogs*, deep venous plasma

C-peptide levels were similar between treatments during the basal period (0.34 ± 0.08

and 0.40 ± 0.08 ng/ml, saline and Ex-9, respectively) and during the infusion of saline or

Ex-9 (0.50 ± 0.11 and 0.39 ± 0.12 ng/ml, t=30 min), prior to the meal (Table 6.1). Deep

venous plasma C-peptide levels after administration of the meal (45 to 200 min) were

1.71 ± 0.31 ng/ml with saline infusion but significantly greater with Ex-9 infusion ($2.18 \pm$

0.36 ng/ml, p=0.01) (Table 6.1).

Plasma Glucagon Levels. In the *normal dogs*, arterial plasma glucagon levels were similar between treatments during the basal period (47 ± 5 and 41 ± 7 pg/ml, saline and Ex-9, respectively), with no change during the infusion of saline or Ex-9 (44 ± 3 and 40 ± 6 pg/ml, t=30 min), prior to the meal (Table 6.1). After administration of the meal (45 to 200 min), glucagon levels were 44 ± 5 pg/ml with saline infusion and 46 ± 9 pg/ml with Ex-9 infusion (Table 6.1). In the *insulin resistant dogs*, deep venous plasma glucagon levels were similar between treatments during the basal period (-20 to 0 min) (43 ± 6 and 42 ± 4 pg/ml, saline and Ex-9, respectively) and during the infusion of saline or Ex-9 (43 ± 14 and 41 ± 5 pg/ml, t=30 min) prior to the meal (Table 6.1). Deep venous plasma glucagon levels after administration of the meal (45 to 200 min) were 53 ± 6 pg/ml with saline infusion and 52 ± 10 pg/ml with Ex-9 infusion (Table 6.1).

Plasma FFA Levels. In the *normal dogs*, arterial plasma FFA levels were similar prior to both treatments during the basal period (884 ± 143 and 773 ± 106 μmol/l, saline and Ex-9, respectively), with no change during the infusion of saline or Ex-9 (912 ± 117 and 770 ± 105 μmol/l, t=30 min) prior to the meal (Table 6.1). After administration of the meal (45 to 200 min), FFA levels were 247 ± 82 μmol/l with saline infusion and 212 ± 76 μmol/l with Ex-9 infusion (Table 6.1). In the *insulin resistant dogs*, deep venous plasma FFA levels were similar between studies during the basal period (731 ± 90 and 842 ± 81 μmol/l, saline and Ex-9, respectively) and during the infusion of saline or Ex-9 (738 ± 36 and 758 ± 74 pg/ml, t=30 min) prior to the meal (Table 6.1). Deep venous plasma FFA levels after administration of the meal (45 to 200 min) were 228 ± 65 μmol/l with saline infusion and 276 ± 66 μmol/l with Ex-9 infusion (Table 6.1).

Net Hepatic Glucose Uptake. In *normal dogs*, the hepatic glucose load was 34.8 ± 3.1

and 30.0 ± 2.2 mg/kg/min prior to the meal (-20 to 30 min) and 56.9 ± 4.7 and 66.1 ± 4.3

mg/kg/min after the meal (45 to 200 min) with saline and Ex-9 infusion, respectively

(Figure 6.5A). NHGB was in a state of output prior to the meal (2.4 ± 0.2 and 1.6 ± 0.3

mg/kg/min, average -20 to 30 min, saline and Ex-9, respectively), and switched to uptake

after meal administration (-3.2 ± 1.3 and -3.4 ± 0.8 mg/kg/min, average 45 to 200 min,

saline and Ex-9) (Figure 6.5B). Hepatic fractional extraction was similar with the two

treatments prior to (-0.07 ± 0.01 and -0.06 ± 0.01, average -20 to 30 min, saline and Ex-9,

respectively) and after meal administration (0.05 ± 0.02 and 0.05 ± 0.01, average 45 to

200 min, saline and Ex-9, respectively) (Figure 6.5C). Hepatic fractional extraction as a

ratio to sinusoidal insulin levels were assessed at limited time points, due to limited portal

sampling of insulin. There was no difference in this ratio in prior (-0.0036 ± 0.0004 and

0.0043 ± 0.0012, saline and Ex-9) or after the meal (0.0017 ± 0.0009 and 0.0007 ±

0.0005, saline and Ex-9) (Figure 6.5D). Net hepatic glucose uptake was not assessed in

the *insulin resistant dogs*.

Nonhepatic Glucose Uptake. In *normal dogs,* nonhepatic glucose uptake (Non-HGU)

was similar in both studies during the basal period (2.1 ± 0.4 and 1.2 ± 0.4 mg/kg/min,

saline and Ex-9, respectively), with no change in response to either saline or Ex-9

infusion (2.1 ± 0.3 and 1.1 ± 0.3 mg/kg/min); however, after the meal, Ex-9 infusion

resulted in significantly greater Non-HGU (4.1 ± 1.1 vs. 7.5 ± 2.0 mg/kg/min, saline vs.

Ex-9, average 45 to 200 min, p=0.049) (Figure 6.6A). Nonhepatic glucose clearance was

similar between the two treatments prior to the meal (2.4 ± 0.3 and 1.5 ± 0.3 ml/kg/min,

average -20 to 30 min, saline and Ex-9, respectively) (Figure 6.6B). After administration
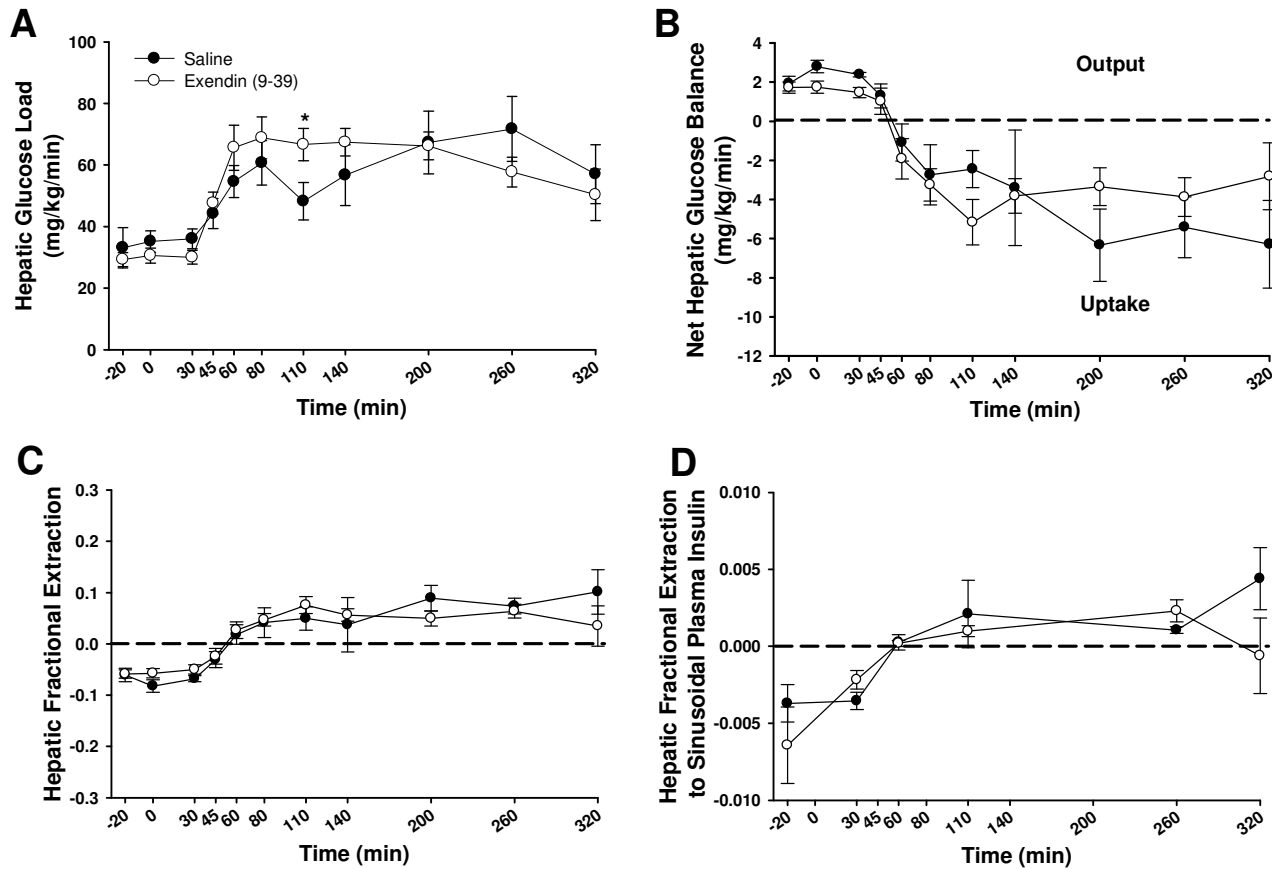
Figure 6.5 Hepatic glucose uptake in normal dogs during the basal period (-20 to 0 min), experimental period prior to meal (0 to 30 min), and experimental period post meal (30 to 320 min). *A*: Hepatic glucose load. *B*: Net hepatic glucose balance. C: Hepatic fractional extraction. *D*: Ratio of hepatic fractional extraction to sinusoidal insulin. Data are mean ± SE. *: $P < 0.05$ compared to saline infusion.
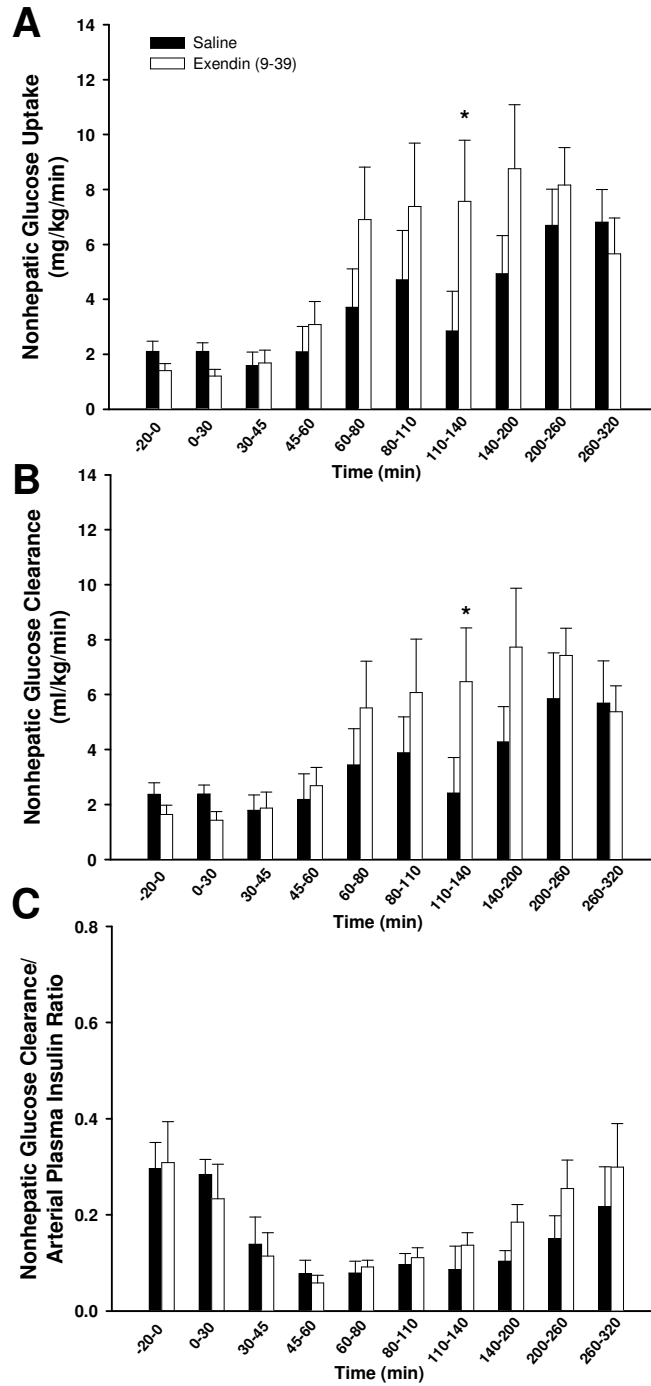
Figure 6.6 Nonhepatic glucose uptake in normal dogs during the basal period (-20 to 0 min), experimental period prior to meal (0 to 30 min), and experimental period post meal (30 to 320 min). A: Nonhepatic glucose uptake. *B*: Nonhepatic glucose clearance. *C*: Ratio of nonhepatic glucose clearance to arterial plasma insulin. Data are mean over time period ± SE. *: P < 0.05 compared to saline infusion.

of the meal (45 to 200 min), average nonhepatic glucose clearance was 3.5 ± 1.0 ml/kg/min with saline infusion and 6.4 ± 1.8 ml/kg/min with Ex-9 infusion (Figure 6.6B). The ratio of nonhepatic glucose clearance to arterial plasma insulin was not different in the two treatments prior to meal delivery (0.29 ± 0.03 and 0.27 ± 0.08 ml/kg/min / µU/ml, -20 to 30 min, saline and Ex-9, respectively), but after meal delivery (45 to 200 min) the ratio with Ex-9 infusion was significantly greater than with saline infusion (0.14 ± 0.02 vs. 0.09 ± 0.02 ml/kg/min / µU/ml, Ex-9 vs. saline, p=0.03) (Figure 6.6C). Nonhepatic glucose uptake was not assessed in the *insulin resistant dogs*.

Hindlimb Blood Flow and Glucose Utilization. Hindlimb glucose balance was assessed in a subset of 3 *normal dogs*. There was no difference in average hindlimb blood flow prior to or after meal administration (Table 6.2). There was no difference in hindlimb glucose uptake between treatments prior to the meal (2.4 ± 0.6 and 3.9 ± 1.7 mg/min, -20 to 30 min, saline and Ex-9); however, after delivery of the meal (45 to 200 min), when animals received Ex-9 (17.2 ± 1.8 mg/min), they had significantly greater hindlimb glucose uptake than when they received saline (9.5 ± 0.9 mg/min, p=0.029) (Figure 6.7A). There was no difference in average hindlimb clearance between studies prior to the meal (2.8 ± 0.7 and 4.5 ± 2.0 ml/min, -20 to 30 min, saline and Ex-9) (Figure 6.7B). After delivery of the meal (45 to 200 min), when animals received Ex-9 (14.0 ± 2.1 ml/min), they had significantly greater hindlimb clearance than when they received saline (8.2 ± 1.5 ml/min, p=0.015) (Figure 6.7B). The ratio of hindlimb glucose clearance to arterial plasma insulin was not different between treatments before (0.39 ± 0.11 and 0.50 ± 0.16 ml/min / µU/ml, -20 to 30 min, saline and Ex-9) or after the meal (0.28 ± 0.19 and
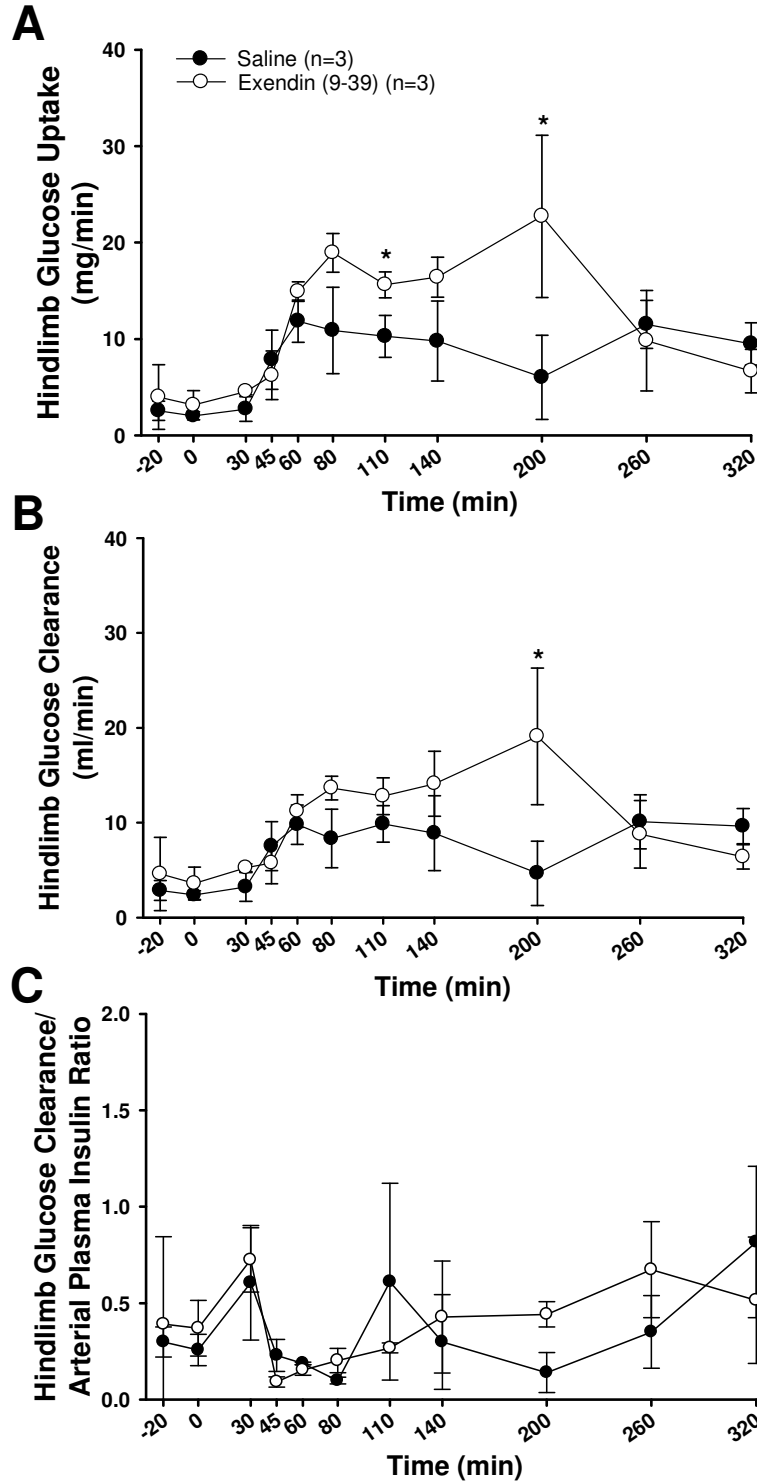
Figure 6.7 Hindlimb glucose utilization in a subset (n=3) of normal dogs during the basal period (-20 to 0 min), experimental period prior to meal (0 to 30 min), and experimental period post meal (30 to 320 min). *A*: Hindlimb glucose uptake. *B*: Hindlimb glucose clearance. *C*: Ratio of hindlimb glucose clearance to arterial plasma insulin. Data are mean ± SE. *: P < 0.05 compared to saline infusion.

0.32 ± 0.09 ml/min / μU/ml, 45 to 200 min, saline and Ex-9) (Figure 6.7C). Hindlimb balance was not assessed in the *insulin resistant dogs*.

Discussion

The purpose of Specific Aim IV was to determine if endogenously secreted GLP-1 has a role in postprandial glucose regulation in the dog. To induce endogenous GLP-1 secretion, a liquid mixed meal was administered orally to both normal and insulin resistant dogs. Each dog was given a meal twice, 30 min after the start of either saline or a GLP-1R antagonist, exendin (9-39) (Ex-9), infusion. The rate at which Ex-9 was infused (500 pmol/kg/min) has been shown in humans to fully block the insulin secretory effect of GLP-1 and does not have agonist effects (294).

The circulating GLP-1 levels were increased in response to the meal in both the normal and insulin resistant dogs; however, levels tended to be greater with Ex-9 infusion than with saline infusion (Table 6.1). This effect has also been observed with Ex-9 infusion in humans (295). Increased GLP-1 level in the presence of Ex-9 could be due to: 1) differences in nutrient load to the gut which would result in differences in nutrient contact with the L cell, 2) negative feedback with inhibition of GLP-1 signaling, 3) a decrease in endocytosis of the peptide with interaction at the GLP-1R, or 4) any combinations of these possibilities. Evaluation of postprandial GLP-1 levels also indicates that the deep venous plasma GLP-1 levels in the insulin resistant dogs are drastically decreased when compared to arterial plasma GLP-1 level in the normal dogs (Table 6.1). This may be directly due to differences in sampling site, or it could be a consequence of the induction of insulin resistance.

Each meal administered to the dogs was spiked with acetaminophen. Acetaminophen is not absorbed by the stomach, but it is absorbed by the gut directly upon leaving the stomach; therefore, circulating acetaminophen levels can be used to quantify gastric emptying. When GLP-1 action was blocked, in both the normal and insulin resistant dogs, acetaminophen levels were higher for approximately 3-4 h after the meal was given (Figure 6.1A, B). In light of the acetaminophen data, we analyzed the response between t=45 min, the first sample taken after meal administration, and t=200, the last sample in which the acetaminophen levels were greater with Ex-9 than saline in both the normal and insulin resistant dogs. The acetaminophen Δ AUC (45 to 200 min) was significantly greater in normal dogs and tended to be greater in insulin resistant dogs when Ex-9 was infused rather than saline (Figure 6.1C, D). Blocking GLP-1 action thus resulted in a decrease in acetaminophen Δ AUC by $18 \pm 10\%$ in the normal dogs and $32 \pm 20\%$ in the insulin resistant dogs. This indicates that endogenous GLP-1 secretion slows gastric emptying for about 2 h and that this mechanism is intact in the insulin resistant dogs.

The question thus arises as to how the modest increase in GLP-1 levels seen in normal dogs, and the dramatically muted augmentation of circulating GLP-1 levels seen in the insulin resistant dogs (Table 6.1) could slow gastric emptying. It is possible that a physiological elevation of GLP-1 in a specific region near its site of release may initiate its postprandial effects and that dramatic elevations in circulating GLP-1 levels are not required. Consistent with the explanation that GLP-1 initiates its effects in a paracrine action close to its site of secretion is the belief that half of GLP-1 secreted is degraded prior to reaching the portal vasculature (58). CCK acts in such a manner to control

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gastric emptying and feeding behavior. It interacts with its receptor at the vagal afferents which terminate in the region of the basolateral membrane in close proximity to entero-endocrine cells (292). It is known that the effects of GLP-1 on gastric motility and acid secretion in humans are dependent on vagal innervation (296), and it has been suggested that in pigs GLP-1 initiates this mechanism by decreasing afferent vagal firing from the gut (211), opposite of the effect observed in the hepatic portal vein in rats (96). It is likely that after a meal GLP-1 is secreted into the vasculature, interacts with its receptor at vagal nerve endings in a paracrine fashion, thereby inhibits parasympathetic flow, and results in slowed gastric emptying.

The effect of slowed gastric emptying on glycemia on two parameters related to glucose absorption were assessed in the normal dogs. Net gut glucose output (7.6 ± 1.1 vs. 11.2 ± 2.2 mg/kg/min, 45 to 200 min, saline vs. Ex-9) and net splanchnic glucose output (4.5 ± 1.0 vs. 7.8 ± 2.0 mg/kg/min, 45 to 200 min, saline vs. Ex-9), were both elevated as a result of an increased rate of gastric emptying when GLP-1 action was blocked (Figure 6.2). It is evident that accelerated gastric emptying contributed to increased glucose absorption as a result of increased glucose load to the gut, but at this point it is unclear if GLP-1 action had a direct effect on the transit of glucose from the gastrointestinal tract into the vasculature of the gut, per se. Increased glucose absorption, due to the accelerated rate of gastric emptying in the early post-meal period, resulted in increased peripheral glycemia. In both the normal and insulin resistant dogs, when GLP-1 action was blocked, average arterial plasma glucose levels and the Δ AUC tended to be greater (Figure 6.3). This occurred over the same duration in which gastric emptying was increased (45 to 200 min). The Δ AUC of plasma glucose levels were increased 19 $\pm$

12% in normal dogs and 37 ± 12% in insulin resistant dogs, similar to the changes observed in Δ AUC of acetaminophen levels over the same time period. This supports the concept that the effect of GLP-1 on gastric emptying contributes to its role in postprandial glucose regulation.

Although GLP-1 is highly touted for its ability to augment insulin secretion, the data shown in relation to our previous aims (268; 280), along with work done by others (49; 237), have failed to observe this effect in dogs. When GLP-1 action was blocked in normal dogs, elevated glucose levels were accompanied by slightly greater arterial plasma insulin levels (Figure 6.4A). The insulin levels correlated with the changes in the plasma C-peptide levels (Table 6.2). In the insulin resistant dogs, when the GLP-1R was blocked the increased glycemia resulted in significantly greater deep venous plasma insulin levels (Figure 6.4B). Again this tended to correlate with increased C-peptide levels (Table 6.1). Antagonism of the GLP-1R might have been expected to limit insulin secretion; however, differences in glycemia confound the data interpretation. In order to circumvent this problem, the ratio of the plasma insulin to plasma glucose level was calculated. This ratio was not different between saline and Ex-9 infusion within either the normal or insulin resistant cohorts (Figure 6.4C, D). Therefore, the small differences in insulin levels that were evident in both the normal and insulin resistant dogs were due to differences in plasma glucose levels and not to a consequence of the inhibition of the GLP-1R. In addition, there was no difference in glucagon levels between treatments in either the normal or insulin resistant dogs (Table 6.1) It can therefore be concluded that the primary role of endogenously secreted GLP-1 in postprandial glucose regulation in

the dog is not an incretin effect. This is consistent with our earlier data (268; 280), and the work of others (237), in which GLP-1 was elevated by a physiologic amount.

Next, we evaluated the direct effect of GLP-1 on glucose uptake. Increased glucose absorption in the presence of GLP-1R blockade resulted in an increase in hepatic glucose load and slightly greater net hepatic glucose uptake. When hepatic fractional extraction was calculated, however, there was no difference between treatments (Figure 6.5C), indicating that GLP-1 did not have a detectable effect at the liver, per se. Likewise, there was no difference in the hepatic fractional extraction to sinusoidal insulin ratio (Figure 6.5D). The increased circulating glucose levels which occurred in response to Ex-9 treatment resulted in greater nonhepatic glucose uptake and clearance (Figure 6.6A, B). The ratio of nonhepatic glucose clearance to the arterial plasma insulin level was not different upon evaluation of the time course (Figure 6.6C), but with Ex-9 treatment the average ratio was greater directly after the meal (45 to 200 min), probably an artifact of quickly changing insulin and glucose levels, which were both greater with the GLP-1R blocked. Further analysis of nonhepatic glucose utilization was conducted in a subset of the normal dogs with evaluation of hindlimb glucose uptake and clearance, both which were significantly greater with GLP-1 action blocked. However, there was no difference between treatments when the ratio of hindlimb glucose clearance to arterial plasma insulin was calculated (Figure 6.7). Therefore, as far as we can tell, increased glucose utilization at the hindlimb was the direct result of increased glycemia and the accompanying increase in insulin secretion.

The effect of GLP-1 on postprandial gastric emptying is in agreement with findings in humans (293; 294). In fact, some claim that this is the primary effect by

which GLP-1 regulates glycemia after a meal in humans (210; 294). When GLP-1 action

during an OGTT is blocked using Ex-9 in humans, peripheral plasma glucose levels are

elevated, and this is accompanied by proportionately elevated insulin secretion (294),

much like the observations in the current study. However, OGTTs administered to GLP-

$1R^{-/-}$ mice result in elevated glycemic levels due to a deficiency in insulin secretion (107;

297). In addition, when Ex-9 is administered to rats or *ob/ob* mice during an OGTT, the

result is elevated glucose due to inadequate insulin levels (298; 299). This suggests that it

is possible that the dog, as opposed to rodents, may be a more accurate model of

endogenous GLP-1 action in the human.

Others have shown that when a dog is given a meal, circulating GLP-1 levels

reach approximately 10 pM, and insulin secretion is significantly greater than when the

glucose profile is matched with a peripheral glucose infusion; however, when GLP-1 was

infused to match these levels in addition to the isoglycemic glucose infusion, there was

no enhancement of insulin secretion (237). This suggests that an incretin effect does

exist in the dog, but is unlikely the result of increased circulating GLP-1 levels. Our lab

has shown that with intraportally infused exendin-4, a potent agonist for the GLP-1R, to

create circulating levels of ~215 pM, insulin secretion was enhanced (300). In addition,

isolated canine pancreata increase insulin secretion in the presence of 1 nM GLP-1 levels

(93). Taken together, these results indicate that the canine β-cell does respond to GLP-1,

but only at higher levels. It is interesting to note that isolated rat pancreas had increased

insulin secretion with exposure to 0.1 nM GLP-1 (93).

The question thus arises as to what is causing the incretin effect in the dog. In

addition to GLP-1, the canine pancreatic islet also increases insulin secretion in response

to the other recognized incretin hormone, GIP (301). It is also possible that GLP-1 and GIP are working in a synergistic manner, which is not replicated with a GLP-1 infusion, alone (237). Previous work from our lab shows that intraportal delivery of glucose alone may enhance the incretin effect in the dog (279); however, this has not been observed in the previous aim in this thesis. Therefore, the exact mechanism of the incretin effect in the dog is unclear.

In conclusion, endogenously secreted GLP-1 has a role in postprandial glucose regulation in both the normal and insulin resistant dog. GLP-1 released after a meal slows gastric emptying, more than likely mediated by afferent vagal signaling. In turn, slowed gastric emptying lowers the rate of glucose uptake by the gut and, ultimately, circulating glucose levels. Blocking of GLP-1 contact with its receptor did not result in decreased insulin secretion. Differences in glucose uptake were reflective of the co-existing glucose and insulin levels, not a direct effect of GLP-1, per se. GLP-1 sensing and its ability to slow gastric emptying is apparently intact in our insulin resistant dogs, since their data mirrored the results of the normal animals.

CHAPTER VII

SUMMARY AND CONCLUSIONS

It is well-recognized that GLP-1 regulates glucose homeostasis with its contribution to the incretin effect. The aim of the research conducted in this thesis is to further the understanding of the physiological role of GLP-1 in glucose regulation.

The incretin effect is present in the dog (237), and GLP-1 infusion into canine pancreata results in increased insulin secretion (93); however, in the current studies, there was no effect of a physiological increase in GLP-1 levels on pancreatic hormone levels. Under various conditions, including both portal and peripheral glucose delivery, intraportal infusion of GLP-1 to create a physiological rise in its plasma levels did not alter either insulin or glucagon levels in plasma. This indicates that a rise in the peptide in the periphery similar to that observed after an OGTT (177) does not induce insulin secretion in the dog. Although it is consistent with what others have found in this species (49; 237), the question is still raised as to why an elevation of the peptide at the β-cell would not enhance insulin secretion. It is possible that variations exist at the β-cell itself which may result in species differences. Homology of the GLP-1R is not completely conserved among species (76; 77); so therefore, it is possible that disparity among species in specificity or number of the GLP-1R at the β-cell could result in alterations of effects of the peptide. Such differences may be reflected in the dose response curve of insulin secretion to GLP-1 level. For example, the minimum concentration for increased insulin secretion in the canine pancreata was ten-fold that required to observe increased insulin

secretion in the perfused rat pancreas (93). In addition, it takes a very high pharmacological dose of exendin-4 to induce a small increase in circulating insulin levels in the dog (300).

It is possible that a physiological elevation of GLP-1 at the β-cell is not the manner in which GLP-1 induces insulin secretion in the dog. In the rat, physiological elevation of GLP-1 in the hepatic portal vein initiates a neural signal which could trigger an indirect effect at the pancreas (96). This indicates that elevation of the peptide closer to the site of its secretion might initiate a neural signal to alter pancreatic hormone secretion. However, as noted earlier, when we elevated intraportal GLP-1 levels under various conditions in the dog, we did not observe an effect of GLP-1 on pancreatic hormone secretion. Once again, this is consistent with what others have found (49; 237). In a further attempt to define the role of GLP-1 on the insulin secretory effect, we induced endogenous GLP-1 secretion with a meal. This increases levels of the peptide in the gut and could possibly initiate an insulin secretory effect of GLP-1 via afferent signals from the gut; however, when the GLP-1R was blocked, there was no difference in insulin secretion, once corrected for the level of glycemia. Therefore, it appears as though a physiological increase in GLP-1 levels is not sufficient to induce the incretin effect in the dog through either direct or indirect mechanisms.

In the current studies, elevations of GLP-1 in the hepatic portal vein (due to intraportal GLP-1 infusion) resulted in significantly greater nonhepatic glucose uptake under conditions of increased insulin, glucose, and the portal signal, when compared to that of a systemic rise in the peptide (due to a hepatic artery infusion). However, net hepatic glucose uptake was significantly greater when GLP-1 was infused into the hepatic

artery rather than into the hepatic portal vein. It is likely that the GLP-1 delivered by either route had a direct effect to enhance net hepatic glucose uptake, which would be in agreement with earlier studies (47). However, it is likely that the direct effect at the liver with intraportal GLP-1 infusion was muted by a preferential enhancement of increased nonhepatic glucose uptake.

To observe the effect of GLP-1 on nonhepatic glucose uptake, intraportal glucose must be present. It is well established that this route of glucose delivery increases net hepatic glucose uptake and decreases nonhepatic glucose uptake (271; 275). It is possible that increased intraportal GLP-1 levels inhibit the signal that is activated by glucose at the brain to induce these effects, and therefore the opposite effects occur. This is consistent with the observation that intraportal GLP-1 infusion did not significantly stimulate net hepatic glucose uptake as it did with hepatic artery GLP-1 infusion, and intraportal GLP-1 delivery increased nonhepatic glucose uptake when compared to intraportal glucose administration alone (saline infusion). In the absence of intraportal glucose infusion, there is no signal to the brain to inhibit, and therefore, no effect. In addition, intraportal GLP-1 and glucose alter afferent vagal firing in opposite directions, either increasing or decreasing impulses, respectively (96; 277), which may account for the opposing effects that the intraportally delivered GLP-1 and glucose appear to have on nonhepatic glucose utilization (271; 272).

There are several explanations for the absence of an effect in the shorter fasted animals. It is possible that glucose uptake is increased (due to increased insulin secretion and increased insulin sensitivity) to a degree in the shorter fasted animals that such a low level of GLP-1 cannot enhance this effect any further. Another possibility is that the

neural signal that is sent by intraportal GLP-1 is not active in the shorter fast, because it has been shown that differences in fasting can deactivate GLP-1 activity in the brain, specifically in regard to its ability to synergize with leptin to have an effect on food intake (291).

The GLP-1R $^{-/-}$ mouse has enhanced glycemia during an OGTT, not due to quickened gastric emptying, but due to lower insulin levels. In contrast, in the dog, as in the human, the effect of GLP-1 on gastric emptying appears to be more important than its effect on insulin secretion (210; 302). We observed the effect of GLP-1 on the rate of gastric emptying in both the normal and insulin resistant dogs. Low circulating levels of GLP-1 in the insulin resistant dogs suggest that GLP-1 may be slowing gastric emptying by a mechanism initiated in the vasculature in close proximity to the L cell. It is possible that GLP-1 is interacting with its receptor in the vagal innervation of the gut, possibly in a manner similar to CCK. In humans, when the GLP-1R is blocked after an oral glucose load, the rate of gastric emptying is quickened, and the effect is great enough to bring about a significant difference in peripheral plasma glucose levels (294). In the dog, we observed a difference in peripheral acetaminophen levels, but not a significant difference in circulating glucose levels. Although not as profound in the dog as in the human, the predominant effect of endogenously secreted GLP-1 on gastric emptying is maintained between these species. This once again indicates that even though GLP-1 homology is maintained among all mammals, the predominant physiological actions may vary between species. Although the species differences do exist, it is impressive that the overall effect of enhancing gluco-regulation is maintained.

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