

PHYSIOLOGY AND PHARMACOLOGY
OF DIABETES THERAPIES IN THE CAT:
INSULIN DETEMIR, INSULIN GLARGINE,
EXENATIDE AND THE INCRETIN EFFECT

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in VMS – Veterinary Clinical Medicine
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

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ABSTRACT

Diabetes mellitus is a common disease in cats. Most diabetic cats depend on insulin therapy to survive but traditional insulin formulations are associated with adverse effects and poor compliance. Novel insulin analogs and incretin-based therapies are more effective and have fewer side effects than traditional therapies.

We studied some of these novel therapies in healthy cats. We used the isoglycemic clamp method to compare the pharmacodynamics of the synthetic insulin analogs, insulin detemir and insulin glargine. An analog-sensitive insulin ELISA was used at the same time to measure exogenous insulin concentrations. We also used the isoglycemic clamp method to study the pharmacodynamics of the GLP-1 mimetic, exenatide. An exenatide-specific ELISA was used for evaluation of exenatide pharmacokinetics. Finally, we studied the incretin effect in cats and compared the effect of glucose, lipids or amino acids on secretion of the incretin hormones glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). The isoglycemic clamp method was used to compare the effects of the 3 treatments on insulin secretion.

We found that insulin detemir and insulin glargine have similar pharmacodynamics in healthy cats. With durations of actions of approximately 12 hours and significant variability in their time-action profiles, their efficacy and safety as once-a-day drugs is questionable. We found that exenatide stimulates insulin secretion in cats in a glucose-dependent manner, but it did not increase glucose tolerability. Its absorption after subcutaneous injection was rapid, but so was its clearance from the blood.

Therefore, the use of exenatide, in its current formulation for treatment of diabetes in cats, is questionable.

Finally, we found that a glucose-stimulated incretin effect does occur in cats, it is probably mediated by GLP-1, and its magnitude is lower than reported in other species. This small incretin effect is probably related to the fact that GIP secretion was not stimulated by oral glucose. GIP secretion was strongly stimulated by oral amino acids and even more so, by oral lipids. GLP-1 secretion was stimulated to a similar degree by all three nutrients.

This dissertation is dedicated to my wife Shir, my son Tom, my family in Israel, and to
my mentor Dr. Thomas Graves

ACKNOWLEDGMENTS

I thank my advisor and mentor, Dr. Thomas K. Graves, for his constant guidance and support. Dr. Graves inspired me as a scientist, as a teacher, and as a veterinarian, but most of all, as a kind-hearted human-being. He taught me more medicine and more science than any other teacher before and I thank him for constantly pushing me to excel. I thank Drs. Olivier Dossin, Amy MacNeill, Karen Campbell and Kelly Swanson for being a part of my committee. I thank my entire committee for guiding me through and for taking the time to review this dissertation. I also thank Dr. Olivier Dossin and Dr. Amy MacNeill for their friendship and for lending an ear when I needed to vent. Dr. Olivier Dossin was also a frequent victim of mine whenever I needed to process out loud my research plans and research results, and for his patience and guidance I am grateful. I also thank Dr. Catherine Dossin and Dr. Markus Rick for their friendship and for patiently sharing office space with me.

I thank the ICU nurses, especially Tara Maggio, who took the time during their busy days and helped with catheter placements. I thank all the caretakers who made sure our research cats are loved and treated like house pets. I also thank Dr. John Scheiderer who agreed on behalf of Nestlé Purina PetCare Company to supply us with free Purina DM, the best food a cat can get. I thank the Winn Feline foundation and the Companion Animal Fund at the University of Illinois who helped funding this work.

I would like to express my deepest gratitude to Tonya Keel Ridge, without her, no doubt, I would not have been able to complete any of my research. For her unending support and friendship and for the many days and nights she helped with my experiments

I am forever grateful. I thank her for the countless hours she spent with our cats, successfully training them to be better research cats, and for the countless hours she spent with me, training me to be a better scientist. For teaching me how to do a glucose clamp, run an ELISA and grow cells in culture, for organizing and taking care of things, for smiling while correcting my mistakes, for smiling while fixing my disasters, for all those things and many more I must have forgotten: Thank you.

Lastly, I thank my wife Shir, Eshet Chayil, who helped me plan and execute my experiments and helped me write grants, papers and this dissertation. Shir, by taking charge of the house and taking care of baby Tom and I, made it possible for me to fully immerse myself in my research and to successfully complete it. אני אוהב אותך שיר.

TABLE OF CONTENTS

	PAGE
LIST OF FIGURES.....	x
LIST OF TABLES.....	xii
LIST OF ABBREVIATIONS.....	xiii
CHAPTER 1: INTRODUCTION.....	1
CHAPTER 2: LITERATURE REVIEW.....	3
<u>Diabetes mellitus in cats</u>	3
<u>Pharmacokinetics and pharmacodynamics of insulin and the isoglycemic clamp method</u>	8
<u>Synthetic insulin analogs and their use in dogs and cats</u>	12
<u>Incretin hormones and the incretin effect</u>	25
<u>Incretin-based therapies for diabetes</u>	44
<u>List of References</u>	49
CHAPTER 3: PHARMACODYNAMICS OF INSULIN DETEMIR AND INSULIN GLARGINE ASSESSED USING AN ISOGLYCEMIC CLAMP METHOD IN HEALTHY CATS.....	76
<u>Abstract</u>	76
<u>Introduction</u>	77
<u>Materials and Methods</u>	79
<u>Results</u>	82
<u>Discussion</u>	85

<u>List of References</u>	89
<u>Tables and figures</u>	92
CHAPTER 4: PHARMACOKINETICS OF INSULIN DETEMIR AND INSULIN GLARGINE IN HEALTHY CATS.....	98
<u>Abstract</u>	98
<u>Introduction</u>	99
<u>Materials and Methods</u>	100
<u>Results</u>	102
<u>Discussion</u>	103
<u>List of References</u>	106
<u>Figures</u>	108
CHAPTER 5: THE INCRETIN EFFECT IN CATS: COMPARISON BETWEEN ORAL GLUCOSE, LIPIDS, AND AMINO ACIDS.....	110
<u>Abstract</u>	110
<u>Introduction</u>	111
<u>Materials and Methods</u>	113
<u>Results</u>	117
<u>Discussion</u>	120
<u>List of References</u>	127
<u>Figures</u>	131

CHAPTER 6: THE GLP-1 MIMETIC EXENATIDE POTENTIATES INSULIN SECRETION IN HEALTHY CATS.....	146
<u>Abstract</u>	146
<u>Introduction</u>	147
<u>Materials and Methods</u>	150
<u>Results</u>	154
<u>Discussion</u>	156
<u>List of References</u>	160
<u>Figures</u>	166
CHAPTER 7: SUMMARY.....	170
<u>List of References</u>	175
AUTHOR'S BIOGRAPHY.....	179

LIST OF FIGURES

FIGURE	PAGE
<u>Figure 3.1.</u> Detemir and glargine time action curves.....	94
<u>Figures 4.1.</u> GIR and insulin concentration during insulin detemir isoglycemic clamps.....	108
<u>Figure 5.1.</u> Expected vs. observed concentrations of GIP in serial dilutions.....	131
<u>Figure 5.2.</u> Expected vs. observed concentrations of GLP-1 in serial dilutions.....	132
<u>Figure 5.3.</u> Blood glucose concentrations during OGTT and IGC.....	133
<u>Figure 5.4.</u> Box plot of the total glucose infused during OGTT, IGC, LIGC and AAIGC.....	134
<u>Figure 5.5.</u> Serum insulin concentrations during OGTT and IGC.....	135
<u>Figure 5.6.</u> Serum total GLP-1 concentrations during OGTT.....	136
<u>Figure 5.7.</u> Blood glucose concentrations during LIGC and IGC.....	137
<u>Figure 5.8.</u> Blood glucose concentrations during AAIGC and IGC.....	138
<u>Figure 5.9.</u> Serum insulin concentrations during OGTT, LIGC and AAIGC.....	139
<u>Figure 5.10.</u> Serum total GIP concentrations during OGTT, LIGC and AAIGC.....	140
<u>Figure 5.11.</u> Serum total GIP concentrations during LIGC and AAIGC in individual cats.....	141
<u>Figure 5.12.</u> Serum total GLP-1 concentrations during OGTT, LIGC and AAIGC.....	145
<u>Figure 6.1.</u> Serum insulin concentrations after exenatide injection and during an isoglycemic clamp.....	166
<u>Figure 6.2.</u> Blood glucose concentrations after exenatide injection and during an isoglycemic clamp.....	167

Figure 6.3. Total glucose infused during IGC and ExIGC in individual cats in paired experiments.....168

Figure 6.4. Exenatide concentrations after a subcutaneous injection at zero minutes...169

LIST OF TABLES

TABLE	PAGE
<u>Table 3.1.</u> Summary statistics of insulin detemir and insulin glargine pharmacodynamic parameters.....	92

LIST OF ABBREVIATIONS

ATP Adenosine-5'-triphosphate

AUC Area under the curve

BG Blood glucose

β -cat/TCF Bipartite transcription factor beta-catenin/T cell transcription factor

cAMP Cyclic adenosine monophosphate

CBC Complete blood count

CI Confidence interval

CV Coefficient of variation

DKA Diabetic ketoacidosis

DM Diabetes mellitus

DPP-4 Dipeptidyl peptidase-4

ELISA Enzyme-linked immunosorbent assay

Epac2 Exchange protein activated by cAMP

FFA Free fatty acids

GI Gastrointestinal

GIP Glucose-dependent insulintropic peptide

GIPR GIP receptor

GIR Glucose infusion rate

GRPP Glicentin-related pancreatic peptide

GLP-1 Glucagon-like peptide 1

GLP-1R Glucagon-like peptide 1 receptor

GLP-2 Glucagon-like peptide 2

GLUT Glucose transporter
GRP G protein-coupled receptor
IGF-1 Insulin-like growth factor 1
IGC Isoglycemic clamp
IDDM Insulin-dependent diabetes mellitus
IU International unit
IV Intra-venous
MPGF Major proglucagon fragment
NEP Neutral endopeptidase
NIDDM Non-insulin-dependent diabetes mellitus
NPH Neutral protamine hagedorn
PC Prohormone convertase
PCV Packed cell volume
PG Proglucagon
PKA Protein kinase A
PZI Protamine zinc insulin
SD Standard deviation
SE Standard error

CHAPTER 1

INTRODUCTION

Diabetes mellitus is common in cats. Although diet change and oral medications can help initially, most diabetic cats depend on insulin therapy to survive. There are some significant problems associated with insulin therapy. Owner compliance is a major problem because twice-a-day injections are required with most commonly used insulin preparations. Weight gain may indicate a good response to therapy initially but can eventually become a problem. Hypoglycemia is a common complication of insulin therapy and can be life-threatening. These side effects have been significantly reduced in human medicine with the introduction of novel treatments for diabetes such as the insulin analogs detemir and glargine, and incretin-based treatments such as exenatide. Insulin detemir and insulin glargine are long-acting insulin analogs. In people, insulin glargine is longer acting than previously used insulin preparations, and is relatively peakless in its effect, whereas insulin detemir has significantly less within-patient variability from day to day. Insulin detemir is also associated with less undesired weight gain and decreased frequency of hypoglycemic events. Because of the smaller risk of side effects associated with insulin analogs compared with insulin formulations that are based on native insulin, insulin analogs can be used to achieve tighter glycemic control. Over time, tight glycemic control is associated with decreased risk of diabetes-related complications. Insulin therapy, however, does not treat the primary defect that causes the disease – it does not directly affect beta cell function.

In contrast to insulin therapy, incretin-based treatments have the potential to reverse the course of the disease. Incretins not only augment insulin secretion but they increase the proliferation and survival of pancreatic beta cells and decrease the rate of programmed cell death that is caused by the disease. There are multiple treatment strategies that take advantage of incretin hormone physiology. Long-acting synthetic GLP-1 mimetics are peptides that must be injected subcutaneously. Oral drugs that prolong the half-life of incretin hormones by inhibiting the enzymes that degrade them are also available. Other oral drugs can stimulate the secretion of incretins by activating specific sensors in incretin-secreting cells of the intestines. These sensors normally respond to specific nutrients in the lumen of the intestine, and the degree of response to different nutrients varies between areas of the gut and between species. Accurate knowledge of the degree of stimulation of incretin secretion by specific nutrients might be exploited for designing novel diets that enhance incretin hormone secretion.

The objective of this research was to study novel treatments for feline diabetes. In particular we studied the synthetic insulin analogs, insulin detemir and insulin glargine, and the GLP-1 mimetic exenatide. We also compared the effect of glucose, amino acids, or lipids on the secretion of incretins and insulin. This information might allow devising dietary manipulations that would increase endogenous incretin secretion and help in the treatment of diabetes in cats.

CHAPTER 2

LITERATURE REVIEW

Diabetes mellitus in cats

Diabetes mellitus (DM) is a disease of glucose homeostasis. In health, glucose homeostasis is achieved through interaction between the pancreas, liver, adipose tissue, muscles and the gastrointestinal tract. The interactions between these organs are orchestrated by nutritional, endocrine and the nervous signals (Shulman et al., 2003). Although complex, abnormal glucose homeostasis (i.e. DM) is usually a result of abnormal function of the pancreatic beta cells and impaired insulin secretion. Abnormalities in other components of glucose homeostasis (e.g. insulin resistance in muscles, obesity, impaired secretion of gastrointestinal hormones, etc.) do not cause diabetes directly, but can aggravate it or unmask a mildly impaired function of pancreatic beta cells. If beta cell function is normal, the capacity to secrete insulin will increase and compensate for the defects in the other components (Ahren and Taborsky, 2003). Treatment of diabetes, therefore, can be directed at any one of the organs that participate in glucose homeostasis, but ultimately, there can be no cure for diabetes unless the dysfunction of beta cells is corrected. The causes of diabetes are multifactorial, complex and not well-understood. There is currently no known cure for any type of diabetes, in cats or in other species. Choosing treatments to manage the disease depends on its type and severity, as well as on cost and convenience.

Classification of diabetes

Diabetes mellitus is a general name for a myriad of syndromes and multiple disease etiologies that are usually classified into a few disease types. In the past, DM was classified into insulin-dependent DM (IDDM) and non-insulin-dependent DM (NIDDM). These were later on replaced by type 1 and type 2 DM respectively. It is now well-recognized, however, that while the etiologies and other key features of type 1 and type 2 DM are different, both can be manifested (at least transiently) as insulin-dependent or non-insulin-dependent (Inzucchi, 2003). The classifications IDDM and NIDDM have also been used in veterinary medicine, and both have been described in cats (Nelson, 2005). Type 1 DM (also called juvenile DM) is an auto-immune disease caused by improper antigen presentation on pancreatic beta cells. Because type 1 DM used to be synonymous with IDDM, and because many diabetic cats are diagnosed as insulin-dependent, it was believed that many cats suffer from type 1 DM. Currently, however, there is no evidence that cats develop type 1 DM except for the rare pathologic description of islet lymphocytic infiltration (Nakayama et al., 1990; Nelson, 2005).

Type 2 DM is a form of diabetes in people that is usually diagnosed at middle-age or older, that is strongly associated with obesity and insulin resistance, and is characterized pathologically by the finding of amyloid deposits in the islet of Langerhans. Genetic predisposition is also a characteristic of type 2 DM (Kahn and Porte, 2003). Another key feature of type 2 DM is impaired beta cell function with residual but declining insulin secretion. This means that while the disease slowly progresses, a decline in insulin sensitivity or in other components of glucose homeostasis can lead to worsening of the overall condition (although not necessarily affecting the

capacity of pancreatic beta cells to secrete insulin). On the other hand, improving insulin sensitivity or any other component of glucose homeostasis can lead to significant improvement in the overall condition, while disease progression is unaltered (Kahn and Porte, 2003). This explains why patients with type 2 DM can often revert from IDDM to NIDDM and back multiple times, especially under circumstances of changes in diet, medications and superimposed illnesses.

Based on the above characteristics of human type 2 DM, and on the characteristics of the disease in cats as described below, diabetes can be classified as type 2 in about 80 – 95% of cats (Rand et al., 2004). Other types, accounting together for only 5 – 20%, are either secondary to diseases causing severe insulin resistance (such as acromegaly and hypercortisolism) or secondary to diseases of the exocrine pancreas (such as pancreatitis and pancreatic adenocarcinoma). Because of the relative rarity of these other DM types, and because they are fundamentally different from type 2 DM in etiology as well as treatment, from here on I will refer to diabetes in cats only in the context of type 2 DM.

Amyloid deposition

Amylin is a peptide hormone that is synthesized in pancreatic beta cells and is secreted together with insulin through the same secretory machinery (Ahren and Taborsky, 2003). Amylin has complementary actions to insulin in controlling glucose homeostasis. In people and in cats, the amino acid sequence of amylin contains a region that makes it prone to polymerization and formation of amyloid. Amyloid is an extracellular, proteinaceous, insoluble deposit exhibiting a fibrillar, beta pleated sheet structure. Amyloid deposits have been shown to have toxic effects on pancreatic beta

cells, and it is suspected that amyloid deposition is one of the factors that leads to progression of type 2 DM, or maybe even causes it. Amyloid deposition is a unique feature of type 2 diabetes and it occurs naturally only in cats and primates. It occurs in more than 90% of human and feline diabetic patients, and it is fairly infrequent in non-diabetics (Henson and O'Brien, 2006).

Epidemiology

Diabetes is most common in domestic long- and short-hair cats. Burmese cats are overrepresented, and many other pure breeds are underrepresented, compared to the incidence in domestic cats (Prahl et al., 2007; Lederer et al., 2009). Increasing age is a risk factor for type 2 diabetes, and most cats are > 8 years of age with a peak incidence between 10 and 13 years of age (Rand et al., 2004; Prahl et al., 2007). In one study, increasing age was the single most important risk factor for diabetes in cats (Panciera et al., 1990). Neutered male cats are at greater risk of developing diabetes than neutered female cats, even when controlled for body weight (Panciera et al., 1990; Prahl et al., 2007). Human diabetes does not have a sex predilection. In a recent study, indoor confinement and low physical activity were identified as independent risk factors for diabetes in cats. This study did not find that diet was an independent risk factor (Slingerland et al., 2009).

Obesity causes insulin resistance; it interferes with glycemic control and confers a high risk of DM in cats (Nelson et al., 1990; Panciera et al., 1990; Biourge et al., 1997; Scarlett and Donoghue, 1998; Appleton et al., 2001; Hoenig et al., 2002; Prahl et al., 2007). Obesity is becoming increasingly prevalent in cats. Based on body condition scoring (BCS), between 25 – 35 % of domestic cats are reported to be overweight or

obese in the United States (Scarlett et al., 1994; Scarlett and Donoghue 1998; Lund et al., 2005). Similar to the obesity epidemic in the human population, environmental factors (e.g. living indoors, diet) predispose cats to obesity (Scarlett et al., 1994; Lund et al., 2005). Importantly, the risk of obesity is highest among middle-aged neutered male cats – the same population group that is at risk of diabetes (Scarlett et al., 1994; Scarlett and Donoghue 1998; Lund et al., 2006).

Insulin sensitivity is defined as the decrease in blood glucose (BG) concentration for a given amount of insulin. Insulin resistance refers to markedly decreased insulin sensitivity. The ability of insulin-resistant individuals to compensate for reduced insulin sensitivity by increasing insulin secretion largely determines the degree to which their glucose tolerance can be prevented from deteriorating (Kahn and Porte, 2003). In individuals in which this balance cannot be maintained, impaired glucose tolerance and overt diabetes ensue.

The frequency of diabetes in cats ranges from 0.5 – 2%, depending on the population studied (Panciera et al., 1990; Rand et al., 2004). Recent evidence suggests that the prevalence is increasing because of an increase in the frequency of predisposing factors such as obesity and physical inactivity (Prahl et al., 2007). Obesity and physical inactivity are also implicated as the causes of increasing frequency of diabetes in people, however, the overall frequency of the disease in people is much higher than in cats (7.8%, <http://diabetes.niddk.nih.gov/DM/PUBS/statistics/#allages>). It is hypothesized that, at least in part, this lower frequency of diabetes in cats is related to different criteria for diagnosis. In people, diagnosis of diabetes is based on fasting hyperglycemia (blood glucose > 130 mg/dl), whereas in cats, it is usually diagnosed once blood glucose has

exceeded the threshold for renal tubular glucose recovery (blood glucose > 290 mg/dl), and when overt clinical signs are evident (Rand et al., 2004). Because type 2 DM is a slowly progressing disease, and because normal fasting blood glucose in people and in cats is essentially the same, it is hypothesized that DM in cats is diagnosed later in the disease process. This explains why most cats are insulin-dependent at the time of diagnosis and why treatments such as diet change and oral hypoglycemic drugs are typically not sufficient to control diabetes in cats (Nelson, 2005).

Pharmacokinetics and pharmacodynamics of insulin and the isoglycemic clamp method

In veterinary medicine, blood glucose curves have been used traditionally to study the pharmacodynamics of exogenous insulin. These curves, however, reflect not only the effect of exogenous insulin but also the effect of endogenous insulin and stress hormones. Because of this major flaw, this method is not used in people. In contrast, the isoglycemic clamp is the gold standard for the study of pharmacodynamics of insulin in people (Heinemann and Anderson, 2004; Heise and Pieber, 2007). With this method, blood glucose concentrations are “clamped” at euglycemia or at sub-euglycemic concentrations by infusing glucose at a changing rate. This allows the study of the effect of injected insulin in a healthy subject with minimum interference from confounding factors such as hypoglycemia, stress hormones, and endogenous insulin secretion (Heinemann and Anderson, 2004; Heise and Pieber, 2007). The clamp method, however, is both labor intensive and expensive.

In healthy or type 2 diabetic subjects, an important component of clamp quality assessment is measurement of C-peptide. C-peptide is the inert cleavage product of proinsulin to insulin. This cleavage occurs inside the secretory vesicles in the pancreatic beta cells, and C-peptide and insulin are secreted in equimolar amounts (Ahren and Taborsky, 2003). Because a variable fraction of insulin is cleared by the liver before the insulin reaches the systemic circulation (and this fraction has a crucial role in glucose homeostasis), the systemic concentrations of insulin are not an accurate measure of secreted insulin. In contrast, C-peptide is not cleared by the liver and can be used as a surrogate measurement of insulin secretion. C-peptide is measured during isoglycemic clamps to verify that endogenous insulin secretion is suppressed to a minimum (Heinemann and Anderson, 2004). The sequence of C-peptide is highly variable between species and a feline-specific C-peptide assay is not available (Hoenig et al., 2006).

In healthy subjects and in type 2 diabetics, endogenous insulin secretion is minimized during the clamp experiment but it is not completely suppressed. This might lead to over-estimation of exogenous insulin action. Ideally, pharmacodynamics of subcutaneously injected insulin should be studied in type 1 diabetics. Because of practical reasons, it is still recommended to perform preliminary pharmacodynamic studies in healthy subjects. In this case, there are a few strategies to cope with the potential of endogenous insulin secretion. First, BG can be clamped at sub-euglycemic concentrations. Depending on clamp quality, this method increases the potential for stress-hormone activation and endogenous glucose production, thus causing an under-estimation of the effect of exogenous insulin. Second, intravenous infusion of regular insulin can be applied, but this might lead to an over estimation of exogenous insulin

action. In both methods, it has been shown in human subjects that complete suppression of endogenous insulin secretion (as reflected by C-peptide measurements) is difficult to achieve (Swinnen et al., 2008). In hyperinsulinemic-euglycemic clamp experiments insulin sensitivity is studied during an intravenous insulin infusion and insulin absorption is, therefore, not a consideration. In hyperinsulinemic-euglycemic clamp experiments a third strategy is used: infusion of somatostatin. This is not a valid strategy for the study of pharmacodynamics of subcutaneously injected insulin because somatostatin alters blood flow, thus affecting absorption of insulin from the subcutaneous depot (Ipp et al., 1987).

Glucose concentrations in venous blood are influenced by local conditions and do not reflect accurately the systemically relevant glucose concentrations. In an ideal isoglycemic clamp, blood glucose concentrations should be measured in arterial blood to reflect the sum of systemic glucose utilization and hepatic glucose output. In clamp experiments in people, blood glucose is measured in “arterialized” venous blood. Arterialization is achieved by continuous warming of the sampled vein by putting the person’s hand in a “hot box” which is kept at 55°C. The warming of the hand causes arteriovenous anastomoses to open, leading to flow of part of the arterial blood directly into the venous system (Heinemann and Anderson, 2004). Sampling of arterialized blood increases the accuracy of the isoglycemic clamp. This method of venous arterialization is clearly not possible in an awake cat. Similarly, direct catheterization of an artery for the purpose of prolonged sampling is not feasible in an awake cat.

Clamp quality in a given experiment can be evaluated by calculating the coefficient of variation of BG during the entire clamp and by calculating the deviation of

target BG from measured BG for each time point in each subject. It is important to note that the BG results themselves are meaningless and should not be used to assess insulin action in the clamp method (Heinemann and Anderson, 2004; Swinnen et al., 2008). This is because the BG at each time point is affected by the action of insulin in the previous 20 – 30 minutes combined with the most recent rate of glucose infusion (which is operator-dependent).

The pharmacokinetics of insulin analogs can be studied in conjunction with the isoglycemic clamp. Specific assays that discriminate circulating exogenous insulin from circulating endogenous insulin and its precursors are required, especially if C-peptide is not measured. If available, the molar concentration of C-peptide can be deducted from the molar concentration of the total measured insulin, thus giving an indirect quantification of exogenous insulin concentration.

Pharmacokinetic data is of limited use if pharmacodynamic data is available. This is because the plasma concentration of insulin is only one of many factors that determine its action. Insulin action also depends on transport and diffusion of insulin through the endothelium and extracellular fluid, and its degradation in the target tissue. Finally, insulin action can vary greatly depending on the sensitivity of the target tissue (Heinemann and Anderson, 2004). All of these factors can vary between different target tissues. In fact, it has been shown that interstitial insulin concentrations, but not plasma insulin concentrations, correlate with glucose-lowering effect of the insulin (Hamilton-Wessler et al., 1999). Transendothelial transport and interstitial insulin concentrations are important factors especially when the pharmacology of albumin-bound insulin analogs (such as insulin detemir) is considered (Ellmerer et al., 2003). Only free detemir,

and not albumin-bound detemir, is available for transendothelial transport (in most tissues) and therefore for action in the tissue. Currently, only total detemir concentrations can be measured in blood, resulting in a pharmacokinetic profile that does not necessarily reflect the active fraction. When studying pharmacokinetic data of insulin detemir and other insulin formulations, it is important to note that because of its low affinity for the human insulin receptor, insulin detemir (Levemir®) is supplied in a molar concentration 4 times higher than the molar concentration of other insulin formulations so that the unit of activity per volume is comparable. Because of the above reasons, it is not recommended to compare pharmacokinetic data of insulin detemir to other insulin formulations (Klein et al., 2007).

Synthetic insulin analogs and their use in dogs and cats

Insulin analogs are artificially altered forms of insulin that differ from native insulin but retain its physiological effects. Recombinant insulin analogs have revolutionized insulin therapy in human diabetes mellitus, and are having an impact on diabetes treatment in veterinary patients as well. Understanding the basics of insulin pharmacology and physiology is key to understanding the properties of synthetic insulin analogs and the rationale for their use.

Insulin physiology

Insulin is secreted by the beta cells of the islets of Langerhans in the pancreas. It reaches the liver through the portal circulation and then enters the systemic circulation and reaches its other target organs – mainly skeletal muscle and adipose tissue. Insulin synthesis and secretion are stimulated predominantly by increases in blood glucose

concentrations, but the degree to which beta cells respond to glucose is modified by a multitude of other factors including nutrients, hormones and neural input. (Ahren and Taborsky, 2003).

Endogenous insulin secretion can be divided into two phases: the “basal” phase, in which insulin is secreted continuously at a relatively constant rate, and the “bolus” phase, in which insulin is secreted in response to nutrients (Owens and Bolli, 2008). The primary role of basal insulin secretion is to limit lipolysis and hepatic glucose production in the fasting state. Post-prandial insulin primarily suppresses hepatic glucose output and stimulates glucose utilization by muscle, thus preventing hyperglycemia after meals (Owens and Bolli, 2008). Post-prandial blood glucose concentration is also largely determined by other factors such as the carbohydrate, fat, and protein content of the meal, gastrointestinal transit time, and the effects of glucagon (Owens and Bolli, 2008). In health, insulin secretion is constantly adjusted to work in concert with these other factors in order to maintain euglycemia. In the war against diabetes, mimicking this highly dynamic process with subcutaneous injections of insulin is a battle best fought with advanced weapons.

Pharmacology of insulin analogs

There is a large body of evidence indicating that tight glycemic control is essential to prevent long term complications of human diabetes (Nathan et al., 2005; Genuth, 2006; Akalin et al., 2009). Intensive treatment protocols to achieve that goal are often associated with side effects such as hypoglycemia and undesired weight gain. The ideal insulin therapy should mimic the physiology of insulin secretion as closely as possible. In veterinary medicine there is no clearly established benefit of tight control of

blood glucose in the normal range, and the standard of care is alleviation of clinical signs while minimizing side effects, rather than achieving sustained euglycemia.

Insulin has a natural tendency to precipitate and crystallize, especially in the presence of zinc. In the pancreatic beta cells insulin is stored as hexamers surrounding molecules of zinc. Insulin hexamers are slow to penetrate capillaries, but when released from the beta cells the zinc is diluted and the hexamers break down to dimers and monomers that are absorbed into the blood stream (Havelund et al., 2004). In older insulin formulations, the tendency of insulin to crystallize is enhanced by modifying the solution (e.g. adding zinc and/or protamine), thus causing precipitation in the vial and at the site of injection (Havelund et al., 2004; Sheldon et al., 2009). Once injected subcutaneously, the zinc is slowly diluted (and protamine slowly degraded), thus releasing insulin into the blood. This strategy has an obvious disadvantage in that insulin has to be re-suspended evenly before being drawn into a syringe, which can lead to inaccuracy in dosing (Kohn et al., 2007). A second disadvantage is that the deprecipitation in the injection site is highly variable and unpredictable, and that can lead to considerable variation in insulin absorption (Havelund et al., 2004; Owens and Bolli, 2008). Third, the older insulin formulations such as lente and NPH have action profiles that are inadequate when trying to mimic normal insulin secretion physiology in human diabetics. The onsets of action are too slow and durations of action are too long to mimic the “bolus” phase; at the same time, insulin action profiles are often too peaked and durations are usually not long enough to mimic “basal” secretion (Havelund et al., 2004; Choe and Edelman, 2007; Owens and Bolli, 2008). A similar problem exists in diabetic dogs and cats. For the typical diabetic pet, twice daily injections of insulin at mealtime is

the standard of care. Using intermediate-acting insulin formulations, this protocol is usually geared towards alleviating clinical signs of diabetes. Achieving tight glycemic control is difficult and increases the risk of hypoglycemia.

Another disadvantage of treatment with traditional insulin formulations is loss of normal liver:periphery insulin concentration gradients (Hordern et al., 2005; Hermansen and Davies, 2007). Inhibition of hepatic glucose output, a major factor in maintaining euglycemia, requires high insulin concentrations in the blood, while inhibition of lipolysis requires much lower concentrations. More than half of the insulin secreted by the pancreas is removed from the bloodstream by the liver before the remainder is circulated to other target organs. When insulin is injected subcutaneously, equal concentrations are delivered to the liver, muscles, and adipose tissue. This accomplishes either appropriate control of hepatic glucose output with inappropriately high concentrations of insulin in adipose tissue (promoting weight gain), or insufficient control of hepatic glucose output leading to poor glycemic control. A synthetic insulin analog that is preferentially targeted to the liver would likely decrease the magnitude of this problem.

Synthetic insulin analogs were designed to mimic physiologic insulin secretion as closely as possible. Intensive insulin therapy protocols in people typically consist of a “bolus” insulin with rapid absorption and ultra-short action given at meal time, and a “basal” insulin given once daily (Choe and Edelman, 2007). These insulin analogs were designed to have more predictable action profiles than older insulin formulations, an important feature in prevention of hypoglycemic events. The synthetic insulin analogs are based on human-recombinant insulin, and are altered biochemically to change their

pharmacological properties. Amino acid substitutions in the B26-B30 region alter the tendency of insulin to crystallize while retaining the ability to activate insulin receptors (Sheldon et al., 2009). All available insulin analogs are supplied as clear solutions and do not need to be re-suspended before use. This reduces inaccuracy in dosing, but insulin analogs can still form hexamers at the site of injection, resulting in some degree of variability in absorption.

Insulin has a mitogenic effect in the body. This effect is mediated by the insulin receptor as well as the IGF-1 receptor (Smith and Gale, 2009). The mitogenic effect of synthetic insulin analogs has been investigated because the modifications to their sequence change their affinity for receptors. Changes in the absolute affinity, as well as the relative affinity to the insulin receptor compared to the IGF-1 receptor, might increase the mitogenicity of a synthetic analog. Few and inconsistent data do exist showing increased risk of developing cancer in people treated with insulin glargine (Weinstein et al., 2009; Hemkens et al., 2009). Contradictory evidence, however, together with obvious benefits of using insulin glargine, have led to the present consensus to support the use of insulin glargine in human diabetic patients (Smith and Gale, 2009; Rosenstock et al., 2009; Home and Lagarenne, 2009; Colhoun, 2009). No clinical data exist regarding the mitogenicity of insulin detemir but one experimental study suggests that it is no more mitogenic than human insulin (Kurtzhals et al., 2000). The affinity of insulin for insulin receptors has been reported in cats and in dogs (Wolfsheimer and Peterson, 1991; Paxton and Ye, 2000; Tolan et al., 2001). IGF-1 receptor affinity has been reported in dogs, (Sukegawa et al., 1987) but not in cats. There is one report of affinity of an experimental synthetic insulin analog for the insulin

receptor in dogs (Kohn et al., 2007) but there are no such reports in cats, nor are there reports of receptor binding studies using commercially available insulin analogs in dogs or cats. As such, there is no evidence to support a claim that any insulin product (natural or synthetic) is safer than another from a mitogenesis standpoint in cats and dogs.

Rapid-acting insulin analogs: Lispro, aspart and glulisine

Historically, a combination of regular insulin and an intermediate-acting insulin was used to replace postprandial insulin in human diabetic patients. However, the action profile of regular insulin after subcutaneous injection may be inadequate for the treatment of diabetes because its absorption is relatively slow and the duration of action is too long (about 5 – 8 hours in people, about 5 hours in cats and dogs) (Plum et al., 2000; Gilor et al., 2008; Rave et al., 2009). Insulin lispro was the first rapid-acting analog to be approved for use in people (Sheldon et al., 2009). The amino-acid sequence of insulin lispro consists of a reversal of proline at the B28 position and lysine at the B29 position. This small change greatly decreases the tendency for association and enhances the rate of absorption. In people, this results in an early onset of action (0.5 - 1 hour) a relatively high peak in activity, and a short duration of action (2 – 3 hours). Thus, subcutaneous insulin lispro is more suited to mimic post-prandial insulin secretion than subcutaneous regular insulin (Sheldon et al., 2009).

In insulin aspart the proline at B28 is replaced with aspartic acid. In insulin glulisine, lysine at B29 is replaced by glutamic acid and on position B3 asparagine is replaced by lysine. Insulin aspart and insulin glulisine have pharmacokinetic and pharmacodynamic profiles similar to insulin lispro (Sheldon et al., 2009). All 3 are used in type 1 and type 2 diabetic people. In type 1 diabetics these insulin analogs have a clear

advantage over regular insulin in reducing the risk of hypoglycemic events (Siebenhofer et al., 2006). In type 2 diabetics, when combined with a basal insulin analog, these rapid-acting insulin analogs provide better glycemic control than regular insulin without increasing hypoglycemic episodes (Mannucci et al., 2009). In one study of human diabetic patients, similar glycemic control was achieved whether insulin aspart was injected 15 minutes before or 15 minutes after initiation of a meal (Brunner et al., 2000). In veterinary medicine, because of the inability of pet owners to predict whether their pet would eat its meal in full, it is recommended that insulin is injected after the meal. Rapid-acting insulin analogs might be of an advantage in this scenario.

There are currently no reports on the use of rapid-acting analogs in the chronic treatment of diabetes in cats and dogs. Insulin lispro has been successfully used in dogs to treat DKA (Sears et al., 2009). In this study insulin lispro was administered intravenously and had similar efficacy as the traditionally used regular insulin. No adverse reactions were seen. There is no clear rationale for preferring insulin lispro over regular insulin for use in constant-rate IV insulin infusions. The biochemical alteration in insulin lispro confers greater dissociation and faster absorption of insulin injected subcutaneously, but both insulin lispro and regular insulin should dissociate immediately when delivered intravenously. This has been observed experimentally in human patients but not to our knowledge in veterinary patients (Horvath et al., 2008). Insulin lispro has also been used experimentally in dogs in one study. It was injected once subcutaneously at a dose of 0.2 U/Kg. Its plasma concentration peaked at 45 minutes and was still high at 3 hours (no further measurements were done). Insulin lispro caused a nadir in blood glucose 2 hours post-injection, and the duration of action was over 3 hours (Matsuo et

al., 2003). Insulin aspart pharmacology has also been studied in dogs, and was reported to have more rapid absorption following subcutaneous injection than regular insulin, but the pharmacokinetics and pharmacodynamics were largely similar to regular insulin (Plum et al., 2000). We are aware of no reports on insulin glulisine in cats or dogs.

Rapid-acting insulin analogs were designed to replace normal bolus phase insulin secretion in people and have a duration of action of 3 hours or less. But what is the “normal” postprandial insulin secretion profile in dogs and cats? In 2 studies in non-diabetic cats, “bolus” phase insulin secretion had a longer duration (over 6 hours in one study and over 12 in the other) and a later and lower peak (occurring at 2-6 hours and reaching 2 – 3 times baseline concentrations compared to over 5 times baseline concentrations in people) compared to the bolus phase in people (Appleton et al., 2001; Mori et al., 2009; Sheldon et al., 2009). In both studies cats were fasted (overnight or for 36 hours) before the meal and were then fed half their daily caloric average over 15-30 minutes. Overall, cats were fed 4 different diets in those 2 studies. If this reflects normal postprandial insulin secretion profile in cats, and if the pharmacologic profile of short-acting insulin analogs in cats is similar to that in people, there would be no advantage to using short-acting insulin analogs in feline diabetes.

Long-acting basal insulin analogs: Insulin glargine

Insulin glargine has 2 arginine residues added to the C-terminus of the B chain at position 30. This modification increases the isoelectric pH of the molecule. A second modification is the replacement of asparagine in position A21 with glycine. This increases the stability of the molecule in acidic pH (Owens and Bolli, 2008). Insulin glargine is soluble at pH 4.0 (in which it is supplied) but in neutral pH (such as in

subcutaneous tissue) it has a strong tendency to precipitate, thus slowing its absorption after injection (Kohn et al., 2007). The precipitation-deprecipitation process, however, introduces a component of variability in absorption, rendering insulin glargine relatively unpredictable in action (Heise and Pieber, 2007). When determined by isoglycemic clamps in people, insulin glargine has a duration of action of over 24 hours and a relatively flat time-action profile. Insulin glargine is commonly used in people as once-daily basal insulin therapy, often supplemented with ultra short-acting insulin analogs given at meal time. Compared to the traditional intermediate-acting formulations, insulin glargine offers similar reductions in glycosylated hemoglobin concentration but with decreased risk of hypoglycemia and greater convenience (Danne et al., 2008; Fakhoury et al., 2008; Monami et al., 2008).

In a small experimental study, the pharmacodynamics of insulin glargine have been studied in dogs and compared to NPH (Mori et al., 2008). Using the isoglycemic clamp technique and with a dose of 0.5 U/kg, insulin glargine had a duration of action of about 18-24 hours with a pronounced peak at 7 hours. NPH had a shorter duration of action (about 12 hours) and peaked at 5 hours. Unexpectedly and in contrast to reports in people, insulin glargine had greater inter-subject variability compared to NPH.

In one study in newly-diagnosed diabetic cats, 8/8 cats became insulin independent when treated with twice-a-day insulin glargine and an ultra-low carbohydrate diet (Marshall et al., 2009). This remission rate was higher than the remission rate for cats treated with PZI (3/8) and Lente (2/8) in the same study. The duration of illness prior to inclusion in the study was not mentioned and the allocation into treatment groups was not random. Also, treatment protocols were not identical

between groups. Lower remission rates were reported in another study in which the goal of treatment was achieving euglycemia (Roomp and Rand, 2009b). In this study 84% of cats that were started on the treatment protocol within 6 months of diagnosis achieved remission while only 35% of cats that were started on the protocol after more than 6 months from diagnosis achieved remission. All cats in this study were fed an ultra-low carbohydrate diet. These remission rates are similar to the results of another study in which cats treated with a variety of insulin formulations other than glargine (mostly PZI) had 68% remission rate when fed a low-carbohydrate diet (Bennett et al., 2006). Only 11 of 31 cats in this study were recently-diagnosed (within 45 days) but there was no difference in remission rates between those cats and others that were sick for over 45 days.

In a small clinical study in cats, once-a-day insulin glargine was compared to twice-a-day Lente in cats fed ultra-low carbohydrate diet (Weaver et al., 2006). In that study both treatment groups experienced improvement in serum fructosamine concentrations, and 16-hour blood glucose curves were improved. Four of the 13 cats of this study experienced remission of diabetes, but only one of these was in the insulin glargine-treated group. Disease duration was not clearly presented in this study. The same group of investigators reported another study in which cats with diabetes were treated with insulin glargine and fed a high-protein / low-carbohydrate diet or a control diet (Hall et al., 2009). Both groups had improved glycemic control, but only 2/12 cats, one in each group, achieved remission. Taken together, these studies suggest that a low-carbohydrate diet in combination with glargine or any other insulin formulation is

clinically useful in treating diabetes in cats. In newly diagnosed diabetic cats, treatment with glargine might be more likely to achieve remission.

Long-acting basal insulin analogs: Insulin detemir

In contrast to insulin glargine, pharmacodynamics of insulin detemir are considered highly predictable in people, with minimal inter- and intra-subject variability (Soran and Younis, 2006; Heise and Pieber, 2007). Insulin detemir has a myristic acid residue (14-carbon fatty acid) replacing threonine at position B30. Instead of the natural, weaker, ionic interactions between insulin molecules, insulin detemir molecules associate through strong hydrophobic interactions between the fatty acids. These fatty acids also bind reversibly to albumin which buffers the concentration of insulin detemir in the blood and tissues, adding to its protracted and more predictable effect. Predictable pharmacodynamics, demonstrated with insulin detemir in human clinical trials, are key to minimizing hypoglycemic events (Heise and Pieber, 2007; Danne et al., 2008; Fakhoury et al., 2008; Monami et al., 2008). The interaction of insulin detemir with albumin also increases the availability of insulin detemir to organs with fenestrated capillaries such as the liver. Relatively high concentrations of insulin detemir are achieved in the liver compared with other target tissues. Thus insulin detemir inhibits hepatic glucose output more effectively, lipogenesis in adipose tissue is decreased, and weight gain is minimized (Hermansen and Davies, 2007; Danne et al., 2008; Fakhoury et al., 2008; Monami et al., 2008). Other acylated long-acting insulin analogs have been described but are not in clinical use. The peripheral action, but not the subcutaneous absorption, of insulin detemir and one other acylated analog (O346) have been studied in dogs (Hamilton-Wessler et al., 1999; Ellmerer et al., 2003). In both studies the insulin

was administered intravenously and in both studies it was shown that the transendothelial transport of the acylated insulin is considerably slower than that of regular insulin. O346 bound so avidly to albumin that its duration of action was estimated to be 2 days in dogs. A thyroxyl-insulin analog that binds to thyroid-hormone binding proteins has also been described (Shojaee-Moradie et al., 1998; Shojaee-Moradie et al., 2000). This insulin analog was hepato-selective in people and in dogs, but its duration of action in people was slightly shorter than the duration of action of NPH insulin.

When determined by isoglycemic clamps in people, insulin detemir has a duration of action of approximately 20 hours (Heise and Pieber, 2007) and it is used commonly as a once-daily basal insulin in human diabetic patients. Although seldom compared side by side, the clinical outcomes of treatment with insulin detemir or insulin glargine are similar. Use of these analogs is associated with similar reductions in glycosylated hemoglobin, with decreased numbers of hypoglycemic events. Insulin glargine may be slightly more effective in reducing glycosylated hemoglobin, and hypoglycemic events may be less common with the use of insulin detemir. Insulin detemir, however, is consistently associated with less undesired weight gain in people (Danne et al., 2008; Fakhoury et al., 2008; Monami et al., 2008).

Insulin detemir vs. insulin glargine in cats

In a study in healthy cats, the duration of action of insulin glargine after a single subcutaneous injection at a dose of 0.5 U/kg was found to be 22 ± 1.8 hours (Marshall et al., 2008). This was based, however, on the return of blood glucose to baseline concentrations during prolonged fasting. While this method of studying insulin pharmacokinetics and pharmacodynamics is common in veterinary research, results of

such studies may not be completely relevant because they do not take into account endogenous insulin and normal physiologic responses to changes in blood glucose. For these reasons, studies of insulin pharmacology are better done by “clamping” blood glucose concentrations to euglycemia, and measuring other indicators of insulin activity. The effects of prolonged fasting were also not taken into account in that study and it is possible that the duration of action of insulin glargine was over-estimated. Interestingly, in the same study, serum insulin concentrations returned to baseline within 6.7 ± 1.3 hours (range 0.6 – 13 hours). We have compared the pharmacodynamics of single 0.5 U/kg injections of insulin detemir and insulin glargine in cats using the isoglycemic clamp method. The onset-of-action of insulin detemir was 1.8 ± 0.8 hours, the end-of-action was reached at 13.5 ± 3.5 hours and there was a significant variation in the time-action profile between cats. Surprisingly, the duration of action of insulin glargine was much shorter than previously reported (11.3 ± 4.5 hours), and like insulin detemir, there was a significant variation in the time-action profile between cats, ranging from curves that were essentially flat to others that had pronounced peaks (Gilor et al., 2010).

Insulin detemir has been compared to insulin glargine in one clinical study in which the goal of treatment was tight glycemic control (maintaining euglycemia with blood glucose concentrations ranging between 50-100 mg/dl) (Roomp and Rand, 2009a; Roomp and Rand, 2009b). Blood glucose concentrations were monitored by owners at home, and the doses of insulin were changed by the owners. All cats in this study were fed high-protein / low-carbohydrate canned diets. Over all remission rate in this study was 67% for insulin detemir and 64% for insulin glargine. Hypoglycemia was common but clinical signs were rarely noticed. Also rare was the occurrence of Somogyi effect.

The median maximum insulin glargine dose was 2.5 IU (range 1.0 to 9.0 IU) compared with a median insulin detemir dose of 1.75 IU (range 0.5 to 4.0 IU). In this study a twice daily regimen of insulin administration was used for both analogs.

Incretin hormones and the incretin effect

K and L cells are enteroendocrine cells dispersed along the epithelium of intestinal tract. Their physiological role is to sense the type and quantity of digested nutrients in the gut. They then secrete incretin hormones as preparatory signals to other remote organs (e.g. brain, pancreas etc.). In the pancreas, the incretin signal is translated to increased sensitivity to the stimulatory effect of glucose. This effect is responsible for the observed difference in insulin secretion between oral and intravenous glucose and is defined as the “incretin effect”; oral glucose leads to much greater insulin secretion compared to intravenous glucose even when blood glucose concentrations are equal (McIntyre et al., 1965; Nauck et al., 1986). Because the stimulatory effect of incretins on the pancreas depends on elevations in blood glucose concentrations, an increase in incretin blood concentration is unlikely to cause hypoglycemia (Holst et al., 2009). The incretin effect is thought to be exclusively mediated by 2 peptide hormones: Glucagon-like peptide-1 (GLP-1) which is secreted from L cells, and glucose-dependent insulinotropic polypeptide (GIP) which is secreted from K cells (Holst et al., 2009). The incretin effect is severely reduced or absent in people with type 2 DM, contributing to glucose intolerance and post-prandial hyperglycemia. In diabetics, the secretion of GIP is normal or slightly reduced but its effect on the pancreas is markedly decreased. In contrast, GLP-1 retains its insulinotropic effects in type 2 DM (at least in

supraphysiologic concentrations), but its secretion is decreased (Nauck et al., 1993; Toft-Nielsen et al., 2001a, Toft-Nielsen et al., 2001b; Holst et al., 2009). Very little is known about incretin hormones and the incretin effect in cats. In the following sections, unless stated otherwise, data from other animal models are reviewed in general. Information regarding incretins in cats will be specified.

Distribution of L and K cells

L and K cells are interspersed in the epithelium of the gut. They are described as open-type cells, reaching the lumen of the gut via a slender apical process. On their basal side they are located in close proximity to the vasculature and neurons of the lamina propria (Baggio and Drucker, 2007). Quantitative immunohistochemical studies in rats, pigs and people have demonstrated that the majority of L cells are located in the ileum and colon, with small numbers in the distal jejunum (Kauth and Metz, 1987; Eissele et al., 1992). In contrast, K cells are concentrated in the duodenum and proximal jejunum (Damholt et al., 1999). A population of K/L cells that contain both GIP and GLP-1 is also present in rats, pigs and people (Mortensen et al., 2000; Mortensen et al., 2003; Fujita et al., 2008). In the mid-jejunum, about two thirds of K and L cells are actually K/L cells. In dogs, the density of L cells increases from the duodenum aborally to the ileum as in other species, however, quantitative analysis revealed that over 70% of L cells are found in the jejunum. Interestingly, K cells in the dog are also concentrated in the jejunum with lesser concentrations in the duodenum. Importantly, over 30% of L cells in the jejunum of the dog are found in close proximity to K cells, suggesting the existence of a paracrine interaction between the two. K/L cells were not described in dogs. For both K and L cells, there is no difference in their density between crypts and

villi (Damholt et al., 1999). In cats, enteroendocrine cells with GIP immunoreactivity were described in the duodenum and jejunum while enteroendocrine cells with glicentin immunoreactivity were described in the ileum and colon (Glicentin or “gut glucagon” is a product of the proglucagon gene as will be discussed later; it was previously thought to be the main hormone product of L cells). No data are available regarding the relative density of K and L cells and their spatial relationship in cats (Larsson and Moody, 1980; Vaillant and Lund, 1986).

Stimulation of secretion of incretin hormones

The main stimulus for secretion of hormones from K and L cells is the presence of nutrients in the lumen of the gut (but not by nutrients in the blood). Hormone secretion takes place in the basolateral side (to the blood). The degree of GIP and GLP-1 stimulation by different nutrients is species-dependent (Deacon, 2005). Fat is more potent than carbohydrates in stimulating GIP secretion in people and dogs but the opposite is true in rodents and pigs (Baggio and Drucker, 2007). Amino acids are weak stimulators of GIP secretion in people but strong stimulators of it in dogs and rats (Pederson et al., 1975; Thomas et al., 1978; Elliott et al., 1993; Wolfe et al., 2000). GIP secretion depends on the rate of absorption of nutrients and not on their mere presence in the lumen of the gut (Baggio and Drucker, 2007). Post-prandial GLP-1 secretion is typically biphasic and is stimulated by ingested lipid, carbohydrate and protein (Elliott et al., 1993, Herrmann et al., 1995). In dogs and rodents, but not in people, it is also stimulated by GIP (Damholt et al., 1999). There are conflicting reports regarding the physiological importance of neural regulation of GLP-1 secretion with some evidence that it is stimulated by the vagus nerve (Kim and Egan, 2008). Different distributions of

GLP-1-secreting cells (L cells and K/L cells) along the intestinal tract in different species determine, in part, the importance of different stimuli of GLP-1 secretion (Damholt et al., 1999; Kim and Egan, 2008).

In people, fat-stimulated GLP-1 secretion necessitates hydrolysis into fatty acids (Beglinger et al., 2010). Monounsaturated fatty acids stimulate GLP-1 secretion more than polyunsaturated fatty acids or saturated fatty acids (Beysen et al., 2002). Fatty acids are sensed by specific G protein-coupled receptors (GPR). GPR40 (FFAR1) and GPR120 are found in L and K cells and they can be activated by saturated and unsaturated medium- and long-chain fatty acids (Parker et al., 2009, Miyauchi et al., 2010). GPR119 has been described recently in L and K cells, and it is activated by phospholipids and fatty acid amides (Overton et al., 2008; Parker et al., 2009). It is likely that continued de-orphanization of GPR's will lead to discovery of other stimulators of incretin secretion (Overton et al., 2008). Drug-delivery systems that target FFA's to GPR120 on colonic L cells and increase GLP-1 secretion have been recently developed as potential treatments for diabetes (Adachi et al., 2008; Morishita et al., 2008). Small-molecule agonists of GPR119 with drug-like properties and oral activity were also described (Overton et al., 2008).

In people and rodents simple sugars are sensed in the luminal surface of the gut via a specific G protein-coupled "sweet taste receptor" that belongs to the same family of taste receptors in the tongue (Jang et al., 2007). Cats lack this sweet taste receptor and they do not seem to sense sweet tastes (Li et al., 2006). Whether or not cats have a different G protein-coupled taste receptor or a different mechanism of sensing carbohydrates by intestinal enteroendocrine cells is unknown. Some studies questioned

the importance of GPR taste receptors in stimulation of secretion from K and L cells (Reimann et al., 2008; Parker et al., 2009). In these studies, glucose stimulated GIP and GLP-1 secretion via glucose metabolism and K_{ATP} channel closure in a mechanism similar to stimulation of insulin secretion in pancreatic beta cells. Nonmetabolizable sugars were still able to promote GLP-1 release via a sodium-glucose cotransporter-dependent mechanism (Gribble et al., 2003, Parker et al., 2009).

Luminal receptors for amino acids have been described, but their presence in K cells or L cells has not been established (Rozenfurt, 2006). In dogs there is some evidence that stimulation of K cells by protein is mediated by pH changes in the duodenum that are secondary to protein digestion in the stomach (Deacon, 2005). Amino acids can also activate secretion of incretins by inducing sodium-dependent inward currents when they enter the cells via specific transporters (Reimann et al., 2004).

In cats, a rapid increase in GLP-1 plasma concentrations after intra-gastric glucose administration has been described (Hoenig et al., 2010). In that study, blood glucose concentrations returned to baseline within 2 hours of glucose administration, suggesting no significant glucose absorption after that time. Plasma GLP-1 concentrations, however, did not return to baseline even after 5 hours in healthy cats, but did return to baseline in obese cats. This is an interesting finding because it might suggest that the stimulatory effect of glucose on GLP-1 secretion persists for hours after glucose has been completely absorbed. Such a prolonged stimulatory effect contradicts the paradigm of incretin biology, i.e. enteroendocrine cells are first-line sensors of nutrients, and incretin hormones serve as preparatory signals for other organs. Such an interpretation of these

data is questionable given the methods used in that study to measure GLP-1 concentrations (as will be discussed later in “Incretins in diabetes and obesity”).

As mentioned before, in people, saturated fatty acids are the weakest incretin secretagogues compared to monounsaturated and polyunsaturated fatty acids (Beysen et al., 2002). Cats are obligate carnivores and, as such, they eat mostly saturated fat. As is the case for glucose, this specialized evolutionary need might have led to a different sensing mechanism for fatty acids or to an altered incretin effect.

Products of post-translational processing of the proglucagon and proGIP genes

The proglucagon gene is expressed in the alpha cells of the endocrine pancreas, in the L cells of the intestine, and in neurons in the caudal brainstem and hypothalamus. Transcription and translation of the proglucagon gene yield a single 180 amino acid precursor protein that is identical in all 3 tissues. Proglucagon undergoes tissue specific posttranslational processing to yield tissue specific peptide profiles (Baggio and Drucker, 2007). In pancreatic alpha cells, proglucagon (PG) is cleaved by prohormone convertase 2 (PC2) into glicentin-related pancreatic peptide (GRPP, corresponding to PG 1-30), glucagon (PG 33-61) and major proglucagon fragment (MPGF, PG 72-158). MPGF contains the sequence of GLP-1 and GLP-2 and it may be cleaved further to generate GLP-1 (1-37) (PG 72-108). GLP-1 (1-37) can go further processing into GLP-1 (1-36) amide. Glucagon is by far the predominant product in alpha cells but some release of GLP-1 (1-37) and (1-36) amide can occur. These latter 2 peptides are biologically inactive but they can cross-react with GLP-1 in immunological assays that detect the C-terminal part of the peptide (Deacon and Holst, 2009).

Posttranslational processing of proglucagon in L cells depends on expression of the prohormone convertase 1/3 (PC 1/3) (Rouille et al., 1997; Ugleholdt et al., 2006). In L cells, processing of proglucagon leaves the N-terminal side of the molecule largely intact, forming glicentin (PG 1-69), small amounts of GRPP (PG 1-30) and oxyntomodulin (PG 33-69). The C-terminal of proglucagon is cleaved to the 2 major products of the L cell: GLP-1 (7-37) (PG 78-108) and GLP-2 (PG 126-158) (Deacon and Holst, 2009). GLP-1 (7-37) is then processed into GLP-1 (7-36) amide. Although both GLP-1 (7-37) and GLP-1 (7-36) amide are biologically active, immunological assays that detect the C-terminus of GLP-1 can detect only one of the two forms (Orskov et al., 1993; Deacon and Holst, 2009). Although the amino acid sequence of GLP-1 is 100% homologous in all mammals in which it has been studied (including rat, hamster, cow, Guinea pig, human being, and pig [Bell et al., 1983; Lopez et al., 1983; Heinrich et al., 1984; Seino et al., 1986; White and Saunders, 1986; Orskov et al., 1989]), the relative concentrations of its 2 active forms differ between species (Pridal et al., 1995). In people, GLP-1 is mostly processed to the (1-36) amide form whereas in dogs, pigs and rodents the 2 forms are secreted in variable ratios (Pridal et al., 1995; Deacon and Holst, 2009). Immunological assays that detect a more central region of the molecule will detect both active forms, but they also detect the inactive forms that are secreted from the pancreas (1-37 and 1-36 amide) as well as active GLP-1 that was degraded to its inactive forms GLP-1 (9-37) and GLP-1 (9-36) amide (the latter 2 will be described later).

Glucagon is the major counterregulatory hormone to insulin. Glucagon regulates hepatic glucose production via activation of glycogenolysis and gluconeogenesis and

inhibition of glycolysis, and is essential for maintaining glucose homeostasis in the fasting state (Baggio and Drucker, 2007).

The physiologic actions of glicentin are not well-defined. Oxyntomodulin inhibits gastrointestinal secretion and motility, stimulates pancreatic enzyme secretion and intestinal glucose uptake and promotes satiety. GLP-2 stimulates cell proliferation and inhibits apoptosis in the intestinal crypt compartment. It also up-regulates intestinal glucose transport, improves intestinal barrier function, and inhibits food intake, gastric emptying, and acid secretion (Baggio and Drucker, 2007).

GIP gene expression has been detected in the stomach and intestinal K cells in rodents and humans, as well as in the submandibular salivary gland in rats. The mature 42-amino acid bioactive form of GIP is released from its 153-amino acid preproGIP precursor via PC1/3-dependent posttranslational cleavage. The peptides encoded within the GIP N- or C-terminal sequences have no known function. The GIP sequence is highly conserved among species with human, mouse, rat, porcine, and bovine GIP exhibiting more than 90% amino acid sequence homology (Baggio and Drucker, 2007; Deacon and Holst 2009).

Degradation and elimination of incretin hormones

Active GIP (1-42) and active GLP-1 (7-37 and 7-36 amide) are degraded by the enzyme dipeptidyl peptidase-4 (DPP-4) into inactive forms: GIP (3-42), GLP-1 (9-37) and GLP-1 (9-36) amide. DPP-4, also known as CD26, is a serine protease that specifically cleaves dipeptides from the amino terminus of oligopeptides or proteins that contain an alanine or proline residue in position 2, thereby modifying or inhibiting their activity (Baggio and Drucker, 2007). Active GLP-1 (but not active GIP) is also degraded

by the enzyme neutral endopeptidase 24.11 (NEP-24.11), a membrane-bound zinc metallopeptidase. DPP-4 and NEP are ubiquitous in tissues, and DPP-4 is also present in a soluble form in the blood. Notably, DPP-4 is present on the surface of endothelial cells, including those lining blood vessels that drain the intestinal mucosa, which are positioned directly adjacent to the sites of GIP and GLP-1 secretion. Consequently, more than 75% of the GLP-1 that enters the portal circulation already has been inactivated (Hansen et al., 1999). Similarly, about 50% of the remaining active GLP-1 is degraded in the liver. Therefore, only 10-15% of secreted GLP-1 reaches the systemic circulation in its active form. It is therefore widely accepted that for estimation of GLP-1 secretion, measuring the active form is inadequate and the total (active and inactive forms) should be measured (Larsen and Holst, 2005; Deacon and Holst, 2009). Measuring total GLP-1 also better estimates the full effect of the active peptide, including its neurally mediated effects within the gut and portal veins, as well as its direct effect on the pancreas. For estimation of the action of GLP-1 directly on the pancreas, it is accepted that active GLP-1 should be measured (Larsen and Holst, 2005; Deacon and Holst, 2009).

When injected intravenously, active GLP-1 has a half-life of about 1-2 minutes. The inactive GLP-1 is quickly cleared by the kidneys and has a half-life of about 4-5 minutes (Larsen and Holst, 2005). Clearance by the kidneys is not affected by disease states such as obesity and diabetes (Vilsboll et al., 2003). The half-life of active GIP in the blood is similarly short (about 2 minutes in rodents and about 5 minutes in healthy people) (Baggio and Drucker, 2007). As mentioned before, active GIP is not a substrate of NEP and its concentrations are therefore exclusively affected by DPP-4 (in contrast to

GLP-1). Like GLP-1, GIP is also cleared in the kidneys. In contrast to active GLP-1, the kidneys are a major degradation site for active GIP (at least in pigs) and thus, more active GIP escapes the portal system and can have systemic effects (Deacon et al., 2001).

In mice, glucose stimulates secretion of GLP-1 while oleic acid and whey protein increase the concentrations of active GLP-1 by inhibiting DPP-4 but not by stimulating secretion (Gunnarsson et al., 2006). Such modulation of DPP-4 activity has not been described in other species to date.

As mentioned above, GLP-1 (9-36) amide is considered the inactive degradation product of the active GLP-1 (7-36) amide. In a recent study, however, GLP-1 (9-36) amide was found to have a marked inhibitory effect on hepatic glucose production and some insulinotropic effect. These effects were much more pronounced in obese people than in lean (Elahi et al., 2008).

The activity of DPP-4 in healthy cats has been indirectly demonstrated in a recent study. Administration of a DPP-4 inhibitor to healthy cats was associated with increased insulin concentrations and decreased glucagon concentrations after an intravenous glucose challenge (Furrer et al., 2010). The half-life of active GIP and GLP-1 and the relative paracrine *vs.* endocrine effects they might have are unknown in cats. Also unknown is the extent of incomplete post translational processing and release of the inactive 1-37 and 1-36 amide peptides in intestinal and pancreatic tissue as well as the extent of amidation of GLP-1 (7-37) to GLP-1 (7-36) amide. All these variables need to be taken into account when choosing an immunological assay to detect incretin hormones. As mentioned before, assays that detect active GLP-1 and active GIP might underestimate the extent of their secretion in response to stimuli as well as some of their

biological effect. Also, assays that detect active GLP-1 are typically “sandwich” assays that use one antibody to detect the intact N-terminal side and a second antibody that detects the amidated C-terminal. Because the extent of amidation of GLP-1 in cats is unknown, such assays might significantly underestimate the concentrations of GLP-1. Assays that use antibodies directed against the middle region of the peptide (“side-view”) will detect active and inactive forms, including amidated and glycine-extended forms. The disadvantage of “side-view” assays is their cross reactivity with the N-terminal extended GLP-1 (1-37) and (1-36) amide. The plasma concentrations of the latter 2 are insignificant in other species but no data exist regarding these issues in cats (Deacon and Holst, 2009).

The effects of incretin hormones in the pancreas

The primary function of pancreatic beta cells is the secretion of insulin in response to changing concentrations of blood glucose. The primary functions of incretin hormones are stimulation of insulin biosynthesis, stimulation of glucose-dependent insulin secretion and regulation of beta cell mass (Baggio and Drucker, 2007). GIP and GLP-1 are ligands of the G protein-coupled receptors GIPR and GLP-1R, respectively. Both receptors are present on pancreatic beta cells. The effects of GIP and GLP-1 on the beta cell are similar and, for the most part, will be discussed here together.

Activation of GLP-1R and GIPR leads to activation of adenylate cyclase and production of cAMP. Subsequently, cAMP stimulates insulin secretion via PKA-dependent phosphorylation of downstream targets and PKA-independent activation of Epac2. The effects downstream include the following: (1) inhibition of K_{ATP} channels, which leads to cell membrane depolarization; (2) increases in intracellular calcium

caused by influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels, activation of nonselective cation channels, and mobilization of intracellular Ca^{2+} stores; (3) increases in mitochondrial ATP synthesis, which lead to further membrane depolarization; (4) closure of voltage-dependent K^+ (K_v) channels and consequent reductions in K_v currents, thereby preventing cell membrane repolarization; and (5) direct effects on the protein machinery of exocytosis (that occur distal to increases in ATP and intracellular Ca^{2+}), including induction of docking, priming and fusion of vesicles (Baggio and Drucker, 2007; Kim and Egan, 2008). These effects are responsible for the potentiation of insulin secretion in response to an increase in blood glucose. The cAMP signal transduction system is also responsible for 2 other major effects in the beta cell: (1) the synergistic effect of incretins with glucose to promote insulin gene transcription, mRNA stability, and biosynthesis (2) enhancement of the glucose-sensing apparatus by increasing the expression of its components: GLUT-2 and glucokinase. Thus incretins have the potential to replenish beta cell insulin stores, prevent exhaustion of beta cell reserves and increase the sensitivity of beta cells to glucose (Baggio and Drucker, 2007; Kim and Egan, 2008).

The effects of GIP and GLP-1 on insulin secretion are similar and (in sub-maximal concentrations) additive. It is suspected that their final pathway to insulin secretion is a common one. On a molar basis, however, GLP-1 is a more potent insulinotropic agent than GIP in at least 2 orders of magnitude (Kim and Egan, 2008).

For the treatment of diabetes, expansion of beta cells mass is probably the most unique and exciting effect of incretins. Incretins potentiate glucose induced proliferation and differentiation of beta cells by activating the expression of immediate early genes

encoding transcription factors that regulate islet cell proliferation and differentiation. GLP-1R agonists also promote conversion of pancreatic ductal cells of the exocrine pancreas into insulin-secreting, glucose-responsive endocrine-like cells (Hui et al., 2001).

Incretins protect beta cells from apoptosis induced by various cytotoxic agents or dexamethasone (Baggio and Drucker, 2007; Kim and Egan, 2008). They also prevent apoptosis secondary to glucolipotoxicity (Robertson et al., 2004). Interestingly, a 2-week infusion of GIP in diabetic rats reduced significantly beta cell apoptosis, despite severe down regulation of the GIPR. This might suggest that GIP has GIPR-independent effects on beta cells (Kim et al., 2005).

GIPR and GLP-1R undergo rapid homologous and heterologous desensitization and internalization. GLP-1R desensitization has not been observed *in vivo*, however, even after long-term GLP-1R agonist administration.

Exendin (9-39), an N-terminally truncated peptide derivative of exendin-4 (which will be described later), binds the GLP-1R and functions as a specific GLP-1R antagonist (Kim and Egan, 2008).

GIP increases glucagon secretion. GIP receptors have been demonstrated on alpha cells and activation of GIPR by GIP has increased intracellular cAMP and Ca^{2+} . This effect of GIP on alpha cells is typically masked during hyperglycemia when GIP-stimulated insulin secretion decreases glucagon secretion (Kim and Egan, 2008). In contrast to GIP, GLP-1 inhibits glucagon secretion in a glucose-dependent manner, thus reducing hepatic glucose output especially in hyperglycemia. The glucagonostatic effect of GLP-1 is lost when blood glucose concentrations decrease below euglycemia, thus

minimizing the risk of severe hypoglycemia. There is controversy as to whether the action of GLP-1 on alpha cells is direct or indirect. GLP-1 receptors have not been demonstrated convincingly on alpha cells (inconsistent data using outdated techniques). In a recent study using *in-situ* hybridization and triple fluorescence in pancreata from mice, rats and people, GLP-1R expression was restricted to beta cells and pancreatic ductal cells, with no significant expression in alpha cells, delta cells (secreting somatostatin) or F cells (secreting pancreatic polypeptide). Rarely, glucagon, insulin and GLP-1R were colocalized to the same cell and in mice only, there was minimal colocalization of somatostatin and GLP-1R (Tornehave et al., 2008). This study therefore, also calls into question the hypothesis that GLP-1 might be affecting glucagon secretion via somatostatin stimulation. More contradictory evidence to the direct glucagonostatic effect of GLP-1 in alpha cells was found in studies of a glucagonoma cell line transfected with GLP-1R and in isolated alpha cell cultures. In both studies, GLP-1 stimulated glucagon secretion mediated by an increase in cAMP, similar to the effect of GIP (Ding et al., 1997; Dillon et al., 2005).

The indirect glucagonostatic effects of GLP-1 are well-established (Baggio and Drucker, 2007). This effect is mediated at least in part by activation of secretion from beta cells and a paracrine effect of insulin and amylin on glucagon secretion. The necessity of mediation by beta cells was recently questioned, however, when GLP-1 led to inhibition of glucagon secretion in a group of type 1 diabetic patients that were C-peptide negative (Asmar et al., 2010). In that report, it was hypothesized that the glucagonostatic effect of GLP-1 is mediated by somatostatin, despite the recent evidence from Tornehave et al. (2008).

Extra-pancreatic effects of incretin hormones

GIPRs are expressed on adipocytes and GIP seems to play a role as an anabolic regulator of fat metabolism. The anabolic effects of GIP in fat include stimulation of fatty acid synthesis and re-esterification, enhancement of insulin-stimulated incorporation of fatty acids into triglycerides, up-regulation of lipoprotein lipase synthesis, and reduction of glucagon-stimulated lipolysis. In rodent models of obesity, knockout of GIPR or chronic administration of a GIPR antagonist leads to resistance to diet-induced obesity and improved glucose tolerance and insulin sensitivity. In some studies, however, blocking GIP action was associated with decreased glucose tolerance (Kim and Egan, 2008). GIPR is not present in liver or muscle tissue and thus it is hypothesized that by blocking GIPR action, fatty acid uptake and oxidation by these tissues is favored, leading to increased energy expenditure and decreased fat storage in face of positive energy balance.

Physiologic concentrations of GIP inhibit gastric acid secretion in dogs but only supra-physiologic concentrations achieve a similar response in people. This correlates with the fact that GIP secretion is stimulated by acid in the duodenum in dogs but not in people as mentioned before. GIP also inhibits GI motility in supra-physiologic concentrations but it does not have an effect on the rate of gastric emptying (Baggio and Drucker, 2007).

GIP and its receptor are expressed in other tissues including bones, brain, heart, and others. Their effect in those tissues does not seem related to glucose homeostasis or diabetes and therefore will not be discussed here (Kim and Egan, 2008).

GLP-1 is a regulator of appetite, food intake and body weight. Studies in rodents show that GLP-1R agonists reduce short-term food intake when injected peripherally or directly into the CNS. Repeated injections inhibit weight gain. These effects are observed also in lean, obese, and diabetic people. GLP-1 crosses the blood brain barrier and exerts its effect on the CNS directly but it also affects it via stimulation of the vagus nerve in the intestines and in the portal system. Interestingly, some of the effects of leptin and ghrelin on food intake are believed to be mediated through GLP-1 (Kim and Egan, 2008).

In contrast to GIP, GLP-1 is a potent inhibitor of gastric emptying and gastric acid secretion, thus it slows the entry of nutrients into the circulation and improves post-prandial hyperglycemia in healthy as well as diabetic patients (type 1 and type 2). The effect of GLP-1 on the stomach is mostly mediated by the vagus nerve, but some evidence exists for a direct effect (Kim and Egan, 2008).

In some experiments GLP-1 stimulated glucose incorporation into glycogen in muscle and liver as well as lipolysis in adipose tissue. Although GLP-1R expression is detected in canine muscle and adipose tissues (Sandhu et al., 1999), evidence for the presence of GLP-1Rs in human or rodent adipose tissue, liver, or muscle is equivocal. Also, the effect of GLP-1 in muscle, adipose and liver tissue is not mediated by cAMP generation but rather by inositol phosphoglycan generation. Interestingly, the GLP-1R antagonist exendin (9-39) has similar effect to GLP-1 in human and rat myocytes (González et al., 2005, Arnés et al., 2008). These data are inconsistent with a GLP-1R-mediated action in muscle, adipose and liver tissues and it is hypothesized that GLP-1 might affect these tissues via an unidentified receptor (Baggio and Drucker, 2007).

GLP-1 and its receptor are expressed in other tissues including thyroid C cells (secreting calcitonin), heart, blood vessels, and others. Their effects in those tissues do not seem related to glucose homeostasis or diabetes and therefore will not be discussed here.

The incretin effect in diabetes and obesity

As mentioned before, the incretin effect is blunted in type 2 DM. The secretion of GIP is normal or slightly reduced but its effect on the pancreas is markedly decreased. In contrast, GLP-1 retains its insulintropic effects in type 2 DM (at least in supraphysiologic concentrations), but its secretion is decreased (Nauck et al., 1993; Toft-Nielsen et al., 2001a, Toft-Nielsen et al., 2001b; Holst et al., 2009). Although the blunted incretin response clearly contributes to the glucose intolerance in diabetes, it seems to be a secondary process caused by diabetes. This is based on a normal incretin effect in glucose-tolerant first-degree relatives or twins of patients with type 2 DM on the one hand, and a blunted incretin effect in patients with diabetes of other types on the other hand (Holst et al., 2009). In disagreement with this notion, there are now data to support the idea that intensified treatment of diabetes, resulting in near normal blood glucose concentrations, leads to a partial restoration of the incretin effects of GLP-1 and GIP on pancreatic beta cells, but it does not restore the normal secretion of incretin hormones in response to a meal (Hojberg et al., 2008).

More evidence for the role of incretins in development of diabetes is the involvement of the Wnt signaling pathway in diabetes. Stimulation of beta cell proliferation by incretins, as well as the effects of incretins on insulin biosynthesis and secretion, is mediated in part by PKA activation of the bipartite transcription factor beta-

catenin/T cell transcription factor (β -cat/TCF). β -cat/TCF is also crucial in induction of GLP-1 biosynthesis. β -cat/TCF is the ultimate effector of the Wnt signaling pathway and it is encoded by the TCF7L2 gene (Jin, 2008). Polymorphisms in the TCF7L2 gene are strongly associated with reduced incretin action and with a strong risk of type 2 diabetes (Lyssenko, 2007, Pilgaard et al., 2009).

There is some evidence that obesity contributes to attenuation of the incretin effect independently of diabetes although the mechanisms of this phenomenon are unknown. In a recent study, obesity was associated with decreased incretin effect including decreased beta-cell response to GLP-1 but also decreased GLP-1 secretion (Muscelli et al., 2008). In some studies, GLP-1 secretion was normal in obese people but GIP secretion was increased during fasting and early after a meal (Vilsbøll et al., 2005, Salera et al., 1982). In contrast, one study showed decreased GLP-1 secretion in response to oral carbohydrate but normal secretion in response to oral fat in obese patients (Ranganath et al., 1996). In obese mice, an increased amount of dietary fat not only increased the concentration of GIP in plasma and in the intestine but, interestingly, it enhanced intestinal K cell density in upper jejunum (Bailey et al., 1986). In another study, insulin resistant mice on a high fat diet had an exaggerated response to oral glucose with an increased incretin effect. The exaggerated effect was at least partially mediated by an increased sensitivity to GLP-1 (Ahren et al., 2008). In contrast, in healthy people, feeding a high fat diet increased the GIP response to glucose but the incretin effect on the pancreas was not exaggerated (Morgan et al., 1988).

Insulin resistance in itself, regardless of obesity or diabetes, has been associated with abnormal secretion of GIP and GLP-1 (Rask et al., 2001). Increased insulin

concentrations in the insulin-resistant subjects might down-regulate incretin secretion (Rask et al., 2001). Decreased incretin secretion might be the limiting factor on appropriate compensatory responses of pancreatic beta cells to insulin resistance, leading to an insufficient increase in insulin secretion and development of glucose intolerance in early diabetes. The possibility that abnormalities in GLP-1 and GIP secretion do not necessarily result from obesity, but rather contribute to its development, should also be considered because of their roles in appetite control (GLP-1) and increasing insulin sensitivity in adipose tissue (GIP) (Kieffer, 2003).

In cats, glucose-stimulated GLP-1 plasma concentrations were higher in lean vs. obese cats (Hoenig et al., 2010). After gastric glucose administration, GLP-1 concentrations did not return to baseline within 5 hours in lean cats but they did return to baseline in obese cats within 3 hours. GLP-1 concentrations were also a lot more variable in lean compared to obese in that study (Hoenig et al., 2010). These data might suggest that in cats, as previously shown in people, GLP-1 secretion is down-regulated by insulin resistance. The effect of diet however, was not controlled in that study. Cats were maintained on different diet regimens in order to maintain their lean or obese states. Also, GLP-1 secretion could not be assessed accurately in that study because a GLP-1 (7-36) amide ELISA was used. As discussed above, the fraction of GLP-1 (7-36) amide out of the total GLP-1 is unknown in cats and its measurement might significantly underestimate the total amount of GLP-1 that was secreted. It is also unknown whether obesity and/or diet affect the rate of conversion to of GLP-1 (7-37) to GLP-1 (7-36) amide or the action of DPP-4 and the rate of degradation to (9-36) amide and (9-37) (as described previously in mice). Thus it is possible that the stimulating effects of glucose

on GLP-1 secretion in cats are not at all different in obese vs. lean cats and that diet, or obesity or a related factor contribute to different processing of GLP-1 in lean vs. obese cats. Another limitation of that study was the use of benzamidine as an inhibitor of DPP-4 in the collected samples. Specific DPP-4 inhibitors are available and are recommended by the manufacturer of the ELISA used in that study. Benzamidine is not one of those recommended inhibitors and therefore it might not be fully effective. GLP-1 (7-36) amide supposedly has a very short half-life in cats (in other species the activity of DPP-4 in plasma results in a half-life of 1-2 minutes). In the absence of a potent DPP-4 inhibitor, small variations in sample collection might result in large variations in concentrations of the measured peptide (e.g. a sample that was drawn over 10 seconds and then placed on ice might have significantly more active GLP-1 than a sample that was drawn over 60 seconds and then placed on ice). Such variations in collection times are not uncommon when working with cats.

Incretin-based therapies for diabetes

Treatment strategies using the incretin effect – Exenatide

The peptide exendin-4 was first isolated from the poisonous venom of the Gila Monster (*Heloderma suspectum*) (Chia and Egan, 2008; Holst et al., 2009). Exendin-4 is a 39-amino acid peptide that shares only a 53% sequence homology with GLP-1 but its affinity for the GLP-1 receptor is 1000 times greater than the affinity of GLP-1. Unlike GLP-1, exendin-4 is not a substrate for DPP-4 and NEP (Chia and Egan, 2008; Holst et al., 2009). Exenatide is a synthetic exendin-4. Resistant to degradation, exenatide is eliminated by the kidneys and has a half-life of 3-4 hours in people. Its biological effect

lasts about 8 hours after subcutaneous injection and it can be detected in the plasma for up to 15 hours (Kolterman et al., 2005). Multiple studies, both *in vitro* and *in vivo*, have shown that, in general, exendin-4 has the same physiologic effects as GLP-1 in the pancreas, GI tract, and brain (Kim and Egan, 2008). Exenatide lowers blood glucose through multiple mechanisms, including enhancement of glucose-dependent insulin secretion, suppression of excess glucagon secretion, reduction of food intake, and slowing of gastric emptying. Exenatide is associated with improvement in some of the earliest and most fundamental abnormalities of type 2 diabetes: diminished “first-phase insulin response” and proinsulin/insulin ratio. Acute administration of exenatide in type 2 diabetic patients corrects the abnormal insulin secretion pattern after an IV glucose bolus (first phase and second phase insulin responses) and restores the ability of beta cells to respond to rapid changes in blood glucose concentrations (Fehse et al., 2005). Exenatide also improves proinsulin/insulin ratio after 30 weeks of treatment (DeFronzo et al., 2005). Exenatide has been shown to be as effective as insulin glargine in the treatment of DM but with less side effects (e.g. hypoglycemia and weight gain) (Heine et al., 2005; Barnett et al., 2007; Glass et al., 2008). In a 2-year follow-up of patients receiving exenatide, patients achieved sustained and significant reductions in glycosylated hemoglobin, accompanied by significant weight loss (instead of weight gain commonly seen in diabetics receiving insulin) and improvement in serum liver enzyme activity and blood pressure. Most importantly, treatment with exenatide improved beta cell function as measured by homeostasis model assessment of beta cell function (HOMA-B) (Buse et al., 2007). Exenatide has also been used recently to

improve the survival and function of transplanted pancreatic islets in type 1 DM (Ghofaili et al., 2007).

Exenatide has minimal side effects in people. It is mostly associated with nausea and less frequently with vomiting. Infrequently, it might cause hypoglycemia. Severe hypoglycemia (requiring assistance) was reported rarely (only 5 of 2781 patients) and only in patients who also received sulfonylurea drugs. The rate of hypoglycemic events decreased over time. Interestingly, the risk of hypoglycemia was not different in exenatide vs. insulin treatment. Antibodies to exenatide developed in 67% of patients but this did not affect outcome and was not associated with side effects (Amori et al., 2007).

When first discovered, exendin-4 was shown to potentiate amylase release from rat acinar pancreatic cells in response to other hormones such as cholecystokinin. This was shown *ex vivo* and in high doses. Although exendin-4 was implicated as a cause of pancreatitis in one case report, a cause and effect has not been proven and exenatide is still considered a safe drug (Malhotra et al., 1992; Dimarco and Denker, 2006; Amori et al., 2007; Holst et al., 2009).

Recently, a study in rats revealed an unexpected consequence of acute exendin-4 administration (Perez-Tilve et al., 2010). Exendin-4, whether administered into the circulation or into the CNS, caused stimulation of the sympathetic nervous system, leading to hyperglycemia. This effect was independent of the glucose-dependent insulinotropic effect of exendin-4 and it waned with more chronic administration (after 6 days).

Exenatide is a peptide and thus it has to be injected to retain efficacy. A long-acting sustained-release formulation of exenatide has recently been described. It consists

of injectable microspheres of exenatide and poly(D,L-lactic-co-glycolic acid), a common biodegradable medical polymer with established use in absorbable sutures and extended-release pharmaceuticals, that allows gradual drug delivery at controlled rates. This drug is administered in people subcutaneously once weekly. It has been shown in a recent clinical study to be more effective than twice-a-day exenatide in achieving glycemic control with no increased risk of hypoglycemia and with similar reductions in body weight (Drucker et al., 2008; Buse et al., 2010). If this drug can be used in cats as effectively, it will revolutionize the treatment of feline diabetes. Studies of exenatide in cats have not been reported to date.

Treatment strategies using the incretin effect – Other GLP-1 analogs, and DPP-4 inhibitors

GLP-1 analogs:

Liraglutide is a human GLP-1 synthetic analog with 2 amino acid substitutions and a fatty acid acyl group that enables noncovalent binding to albumin, thereby extending the pharmacokinetic profile of the GLP-1 molecule. Liraglutide exhibits a prolonged pharmacokinetic profile after a single injection, and exhibits all of the actions of native GLP-1 (Baggio and Drucker, 2007). Liraglutide (once-a-day) was recently compared to exenatide (twice-a-day). Liraglutide provided significantly greater improvements in glycemic control than did exenatide and was generally better tolerated (Buse et al., 2009). Liraglutide has been used successfully to treat obesity in non-diabetic patients (Astrup et al., 2009).

Albugon (Naliglutide) is a recombinant GLP-1-albumin protein that exhibits a reduced affinity for the GLP-1R, but displays a broad spectrum of GLP-1R-dependent actions in preclinical studies, including inhibition of food intake and gastric emptying and reduction of glycemia excursion after meal ingestion (Baggio et al., 2004).

DPP-4 inhibitors (e.g. sitagliptin, vildagliptin) are administered orally. Vildagliptin and sitagliptin are well-tolerated and not associated with hypoglycemia when used alone. Both agents increase plasma concentrations of GLP-1 and GIP after meal ingestion, enhance glucose-stimulated insulin secretion and reduce ratios of proinsulin:insulin, consistent with an improvement in beta cell function. They are, however, less potent than other oral hypoglycemic drugs (Baggio and Drucker, 2007). In contrast to GLP-1 analogs, DPP-4 inhibitors are not associated with nausea or vomiting but they are associated with weight gain. DPP-4 inhibitors are also associated with increased risk of nasopharyngitis, urinary tract infections and headaches (Amori et al., 2007). Increased risk of infections might be related to the action of DPP-4 in T-cells as a co-stimulatory molecule (CD26).

As mentioned before, a DPP-4 inhibitor has been used experimentally in cats and was effective in enhancing insulin secretion and inhibiting glucagon secretion after an intravenous glucose challenge. Glucagon inhibition was also observed after a meal challenge (Furrer et al., 2010). In that study, the drug was administered subcutaneously and not orally.

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CHAPTER 3

PHARMACODYNAMICS OF INSULIN DETEMIR AND INSULIN GLARGINE ASSESSED USING AN ISOGLYCEMIC CLAMP METHOD IN HEALTHY CATS

Abstract

Background: Insulin detemir and insulin glargine are synthetic long-acting insulin analogs. In people, insulin glargine is longer acting and relatively peakless, while insulin detemir has significantly less within-subject variability. Insulin detemir is also associated with less undesired weight gain and decreased frequency of hypoglycemic events.

Objectives: To compare the duration of action of insulin detemir and insulin glargine in healthy cats.

Animals: Ten young, healthy, neutered, purpose-bred cats

Methods: Randomized, cross-over design. Pharmacodynamics of insulin detemir and insulin glargine were determined using the isoglycemic clamp method following a 0.5 U/kg subcutaneous injection.

Results: There were no significant differences in the pharmacodynamics of insulin detemir and insulin glargine except for onset of action (mean onset \pm SD of insulin detemir 1.8 ± 0.8 hours, mean onset of insulin glargine 1.3 ± 0.5 hours, $P = 0.03$). End of action of insulin detemir was reached at 13.5 ± 3.5 hours and for insulin glargine at 11.3 ± 4.5 hours. Time to peak action of insulin detemir was reached at 6.9 ± 3.1 hours and

for insulin glargine at 5.3 ± 3.8 hours. The time-action curves of both insulin analogs varied between relatively peakless curves in some cats and peaked curves in others.

Conclusion and clinical importance: Insulin detemir and insulin glargine have shorter durations of action than previously reported in people when using the clamp method, but in some cats these insulin analogs may be useful as once-a-day drugs. Peak effects of both insulin analogs are pronounced in some cats.

Introduction

Type 2 diabetes mellitus is common in cats. Although diet change and oral medications may help initially, most diabetic cats depend on insulin therapy to survive (Nelson, 2005; Marshall et al., 2008a). There are some significant problems associated with insulin therapy. Owner compliance is a major problem because twice-a-day injections are required with most commonly used insulin preparations. Weight gain may indicate good response to therapy initially but can eventually become a problem. Hypoglycemia is a common complication of insulin therapy and can be life-threatening (Nelson, 2005). These side effects have been significantly reduced in human medicine with the introduction of newer insulin analogs such as insulin detemir and insulin glargine (Danne et al., 2008; Fakhoury et al., 2008; Monami et al., 2008). These are synthetic human insulin analogs that have been modified chemically to improve their pharmacological profiles. Insulin glargine has 2 arginine residues added to the C-terminus of the B chain at position 30. This modification increases the tendency of the insulin molecules to precipitate in neutral pH, thus slowing its absorption after subcutaneous injection. The precipitation-deprecipitation process, however, introduces a

component of variability in absorption, rendering glargine relatively unpredictable in action (Heise and Pieber, 2007). In contrast, pharmacodynamics of insulin detemir are considered highly predictable in people, with minimal inter- and intra-subject variability (Soran and Younis, 2006; Heise and Pieber, 2007). Insulin detemir has a myristic acid residue (14-carbon fatty acid) replacing threonine at position B30. The protracted absorption after injection is caused by hydrophobic interactions between the fatty acids (Havelund et al., 2004). Those fatty acids also bind reversibly to albumin which buffers the concentration of insulin detemir in the blood and tissues, adding to its predictable effect. Predictable pharmacodynamics, demonstrated with insulin detemir in human clinical trials, are key to minimizing hypoglycemic events (Heise and Pieber, 2007; Danne et al., 2008; Fakhoury et al., 2008; Monami et al., 2008). The interaction of insulin detemir with albumin also increases the availability of insulin detemir to organs with fenestrated capillaries (i.e. liver), thus mimicking a more physiologic state compared to other subcutaneously injected insulin preparations (Hermansen and Davies, 2007). This helps minimize undesired weight gain in diabetic people (Danne et al., 2008; Fakhoury et al., 2008; Monami et al., 2008). When determined by isoglycemic clamps in people, insulin glargine has a duration of action of over 24 hours and insulin detemir has a duration of action of approximately 20 hours (Heise and Pieber, 2007). Both are used effectively as once-daily basal insulin therapy, and are often supplemented with ultra short-acting insulin analogs given at meal time (Danne et al., 2008; Fakhoury et al., 2008; Monami et al., 2008).

In veterinary medicine, blood glucose curves have been used traditionally to study the pharmacodynamics of exogenous insulin. These curves, however, reflect not only the

effect of exogenous insulin but also the effect of endogenous insulin and stress hormones. Because of this major flaw, this method is not used in people. In contrast, the isoglycemic clamp is the gold standard for the study of pharmacodynamics of insulin in people (Heinemann and Anderson, 2004; Heise and Pieber, 2007). With this method, blood glucose concentrations are “clamped” at euglycemia or at sub-euglycemic concentrations by infusing glucose at a changing rate. This allows the study of the effect of injected insulin in a healthy subject with minimum interference of confounding factors such as hypoglycemia, stress hormones and endogenous insulin secretion (Heinemann and Anderson, 2004; Heise and Pieber, 2007). The clamp method, however, is labor intensive and expensive. Based on blood glucose curves, it has been reported that the duration of action of insulin glargine is 24 hours in some cats, but twice-a-day administration has been recommended because of better clinical response (Marshall et al., 2008a; Marshall et al., 2008b). The same studies also demonstrated large variation between cats in response to insulin glargine. The pharmacodynamics of insulin detemir are not reported in cats. The goal of our study was to compare the pharmacodynamics of insulin detemir and insulin glargine in healthy cats, with emphasis on end of action.

Materials and Methods

Animals: Ten young, healthy, purpose-bred cats were used in this study (6 spayed females and 4 neutered males). Cats were group-housed and experiments were performed in AAALAC-accredited facilities and all animal use was approved by the University of Illinois Institutional Animal Care and Use Committee. All cats were acclimatized and socialized for 8 weeks prior to the start of experiments, and extensive

environmental enrichment was provided. This included spending at least 3 hours daily in a 100 sq ft play area with climbing apparatus, elevated perches, and various toys, and 2 - 3 hours daily of interaction with people. Cats were fed commercial cat food^a and monitored daily by physical exams. Body weight was measured weekly. Routine laboratory tests (including CBC, serum chemistry panels, total T4, coagulation panels and urinalysis) were performed at first arrival and just prior to the first clamp experiment in each cat. PCV, total solids and CBC's were repeated prior to the second clamp experiment in each cat. Experiments were performed in ambient temperatures between 19 and 22°C.

Study design: A randomized crossover study design was used. Each cat received 2 subcutaneous injections of 0.5 U/kg of insulin, once with insulin detemir^b and once with insulin glargine^c, on 2 different days, at least 1 week apart (and no more than 4 weeks apart) in random order. To maximize dosing accuracy, 0.3 ml (100 U) insulin syringes were used. The dose was rounded up to the nearest half unit. Insulin was injected on the back, midway between the scapulas. Experiments were always started between 8:00 and 10:00 a.m. on a given day. Cats were fasted overnight for 16 hours prior to each experiment and remained fasted during the experiment.

Blood samples were collected through indwelling jugular catheters^d placed under sedation with dexmedetomidine (0.009 mg/kg), butorphanol (0.22 mg/kg), and atropine (0.022mg/kg), at least one day prior to the experiment. These catheters were maintained by flushing heparinized saline daily. Cephalic catheters^e were placed the day before each experiment and removed at the end of it. These catheters were used exclusively for glucose infusion. For glucose infusion, a 50% dextrose solution^f was diluted with saline

to a 20% solution. Infusion rate was set on a syringe pump. Blood glucose concentrations (BG) were measured with a hand-held point-of-care glucose meter^g that was validated for use in cats in our laboratory (range: 20 – 600 mg/dl, intra-assay CV = 2.46%, correlation with standard hexokinase method: $r = 0.984$, $P < 0.0001$).

Isoglycemic clamp method: Following insulin injection, BG was maintained (“clamped”) at a target isoglycemic concentration for the duration of the experiment by infusing glucose. BG was measured every 5 minutes and then the glucose infusion rate (GIR) was adjusted to achieve the target BG. Baseline BG was determined by averaging 3 - 4 BG measurements taken every 15 - 20 minutes prior to insulin injection. The target BG was defined as $0.9 \times$ baseline BG. Glucose infusions were started when BG reached the target BG. GIR was recorded manually every 5 minutes. The insulin time-action curve was defined as the GIR over time. The onset of action was defined as the time from insulin injection (time zero) until the target BG was first reached (or first increase in GIR). The end of action was defined as the time from insulin injection until GIR stabilized back at zero. The duration of action was defined as [end of action – onset of action]. The graph from each clamp experiment was smoothed using computer softwareⁱ [which calculates a local polynomial regression (in this case of the 4th order) on a series of values (in this case the adjacent 9 neighbors) to determine the smoothed value for each point]. The peak insulin action was defined as the highest GIR of the smoothed data. Time to peak was defined as the time elapsed from insulin injection until peak insulin action. The average time action curves for insulin detemir and insulin glargine were calculated from the raw data and then smoothed. The total metabolic effect was defined as the total amount of glucose infused throughout the clamp.

Statistical analysis: Statistical analysis was performed using computer software^h. Data are reported for the entire group (N = 10) as mean \pm SD unless specified otherwise. The Shapiro-Wilk test was used to assess deviance from normal distribution of data. The coefficient of variation (CV) was calculated for each parameter for each insulin group by dividing the standard deviation with the group mean. Homogeneity of variance was compared between groups using F tests. Differences in ages and body weights of males and females were analyzed using the Mann-Whitney test.

Clamp quality was evaluated by calculating the coefficient of variation of BG during the entire clamp and by calculating the deviation of target BG from measured BG for each time point in all cats. The significance of the deviation from zero was assessed using a one-sample t-test. Pharmacodynamic parameters in the entire study population were normally distributed. Paired t-tests were used to compare means between treatment groups. For each cat, differences between detemir and glargine of a pharmacodynamic parameter were calculated as $X_{\text{detemir}} - X_{\text{glargine}}$. Pearson's r correlation and linear regression were used to assess the association between differences (in individual cats) of onset of action, duration of action and end of action. The Wilcoxon matched pairs test was used to compare means between treatment groups when the data was analyzed separately for males and females. All statistical tests were performed as two-tailed tests and a P value < 0.05 was considered significant.

Results

There were 6 spayed females and 4 neutered males in this study. The mean age for the entire study population (n = 10) was 3.4 ± 0.7 years (range 1.5 – 4.5 years), mean

BW was 5.3 ± 0.95 kg (range 3.7 – 6.9), and the median BCS was 5/9 (range 4/9 – 7/9) with no significant difference between males and females.

After an over-night fast, baseline BG ranged between 67 – 97 mg/dl (mean 77.4 ± 8.0). Target BG ranged between 60 – 79 mg/dl (mean 70.9 ± 7.0). In one cat, the baseline of BG of 97 mg/dl was inconsistent with previous fasting BG and the target was set at 77 mg/dl. Mean CV of BG for the 20 clamps was $15\% \pm 4$. There was no significant difference in CV of BG between insulin detemir and insulin glargine clamps ($P = 0.17$). The mean deviation of target BG from actual mean BG was 0.12 ± 3.09 mg/dl and this deviation did not differ significantly from zero ($P = 0.85$). There was no significant difference between insulin detemir and insulin glargine clamps in the absolute deviation of target BG from actual mean BG ($P = 0.48$).

No side effects were seen in the injection site or systemically after any of the clamp experiments.

Summary statistics of pharmacodynamic parameters are presented in table 3.1. There were no significant differences in the pharmacodynamic parameters of insulin detemir and insulin glargine between males and females and therefore the results are presented for the entire study population. There were no significant differences in the pharmacodynamic parameters of insulin detemir and insulin glargine except for onset of action (mean onset of insulin detemir 1.8 ± 0.8 hours, mean onset of insulin glargine 1.3 ± 0.5 hours, $P = 0.03$). The difference in onset of action between insulin detemir and insulin glargine in individual cats ranged from -0.25 to 1.4 hours. In 7 cats the onset of action of detemir was later than the onset of action glargine but in only 2 of them the difference was greater than one hour (1.3 and 1.4 hours). End of action of insulin detemir

was reached at 13.5 ± 3.5 hours (CV = 26.4%) and for insulin glargine at 11.3 ± 4.5 hours (CV = 40.2%). The difference in end of action between detemir and glargine in individual cats ranged from -4.4 hours to 9.8 hours. In 6 cats the end of action of insulin detemir was longer than that of insulin glargine; those differences were 0.3, 0.5, 3.4, 7.7, 8.4 and 9.8 hours. The differences in end of action were not related to delayed onset of action: The differences in duration of action were similar to the differences in end of action in each cat and there was no significant correlation between onset of action difference and end of action difference ($r = -0.22$, $P = 0.53$) and between onset of action difference and duration of action difference ($r = -0.34$, $P = 0.33$). In contrast, there was a strong association between duration of action difference and end of action difference [$r = 0.99$ (95% CI 0.97 – 1.0), $R^2 = 0.98$, $P < 0.0001$, slope = 1.03 (95% CI = 0.93 – 1.13)].

Time to peak action of insulin detemir was reached at 6.9 ± 3.1 hours (CV = 45.3%) and for insulin glargine at 5.3 ± 3.8 hours (CV = 72.9%) (Table 3.1). The total metabolic effect of insulin glargine was positively correlated with duration of action [$r = 0.64$ (95% CI 0.02 – 0.90), $P = 0.046$] with a similar trend in correlation with end of action [$r = 0.57$ (95% CI -0.09 – 0.88), $P = 0.024$]. The total metabolic effect of insulin glargine was negatively correlated with onset of action [$r = -0.80$ (95% CI -0.95 – -0.36), $P < 0.005$] but did not correlate with peak action [$r = 0.26$ (95% CI -0.45 – 0.76), $P = 0.47$]. These results were consistent with an overall trend towards a peakless time-action profile. In contrast, the total metabolic effect of insulin detemir did not correlate with any of these parameters. For both insulin analogs, however, the individual time-action curves (Figure 3.1) varied between flat and peaked and the shape of the curve did not seem to be insulin dependent or cat dependent.

Discussion

Long-acting basal insulin analogs are designed to approximate the relatively constant secretion of insulin from the pancreas between meals. Physiologically, the main function of this basal secretion is to curb hepatic glucose output. The ideal basal insulin analog should replace this basal secretion but should not exceed it so that hypoglycemia is avoided. This hypothetical ideal insulin analog cannot be studied in healthy subjects by using the isoglycemic clamp method because if hypoglycemia is not induced, GIR is maintained at zero, and no meaningful data can be generated (Swinnen et al., 2008). This illustrates one limitation of the isoglycemic clamp method: Its sensitivity is determined by the target BG. In this study we chose a target BG that is lower than euglycemia because we aimed to decrease bias towards artificial prolongation of perceived insulin action. This artificial prolongation is a potential problem in subjects capable of endogenous insulin secretion (i.e. healthy and type 2 diabetics) because small deviations in BG towards the hyperglycemic range can induce secretion of insulin and prompt a further increase in GIR. When euglycemic clamps are performed to study insulin sensitivity, endogenous insulin secretion is suppressed by administering somatostatin and insulin intravenously. Somatostatin should not be used for the study of pharmacodynamic parameters following subcutaneous insulin administration because it affects subcutaneous blood flow and insulin clearance (Ipp et al., 1987; Swinnen et al., 2008). Artificially long duration of action is also a potential problem considering the effects of fasting on insulin sensitivity and BG concentration. In people, prolonged fasting (24 hours) causes increased insulin sensitivity and decreased BG concentration (Swinnen et al., 2008). This occurs even under euglycemic clamp conditions in which

glucose is infused over 24 hours in patients receiving placebo instead of insulin (Heinemann et al., 2000; Heise and Pieber, 2007; Swinnen et al., 2008). In a recent study in healthy cats, the duration of action of insulin glargine after a single subcutaneous injection at a dose of 0.5 U/kg was found to be 22 ± 1.8 hours (Marshall et al., 2008a). This was based, however, on the return of BG to baseline concentrations during prolonged fasting and without infusing glucose. The effects of prolonged fasting were not taken into account in that study and it is possible that the duration of action of insulin glargine was over-estimated. Interestingly, in the same study, insulin concentrations returned to baseline within 6.7 ± 1.3 hours (range 0.6 – 13 hours), suggesting either a prolonged after-effect of insulin, difficulty measuring insulin glargine, or an over-estimation of the duration of action. These pharmacokinetics data are more consistent with our results of duration of action of insulin glargine (Table 3.1).

Insulin detemir and insulin glargine are used extensively in people as once-a-day basal insulin replacement. Although insulin glargine is longer-acting in people, it is not clinically superior to insulin detemir (Heise and Pieber, 2007; Fakhoury et al., 2008; Monami et al., 2008). Insulin detemir has the advantage of decreased day-to-day variability within subjects (Heise and Pieber, 2007; Klein et al., 2007; Danne et al., 2008). We found no significant differences between insulin detemir and insulin glargine except for longer onset of action of insulin detemir (indicating a more protracted absorption). The difference in onset of action was small and it did not affect the duration of action. In 3 of 10 cats the end of action of insulin detemir was longer by over 7 hours than the end of action of insulin glargine. This may suggest that, in some cats, there

might be a clinically relevant difference between the 2 analogs, but this is only true if the day-to-day effect is fairly constant.

Based on our results, both analogs could be useful as once-a-day drugs in some cats, given a median end of action of over 12 hours (Table 3.1). The combination of positive correlation between total metabolic effect and duration of action, together with lack of correlation between total metabolic effect and peak action suggests that insulin glargine is relatively peakless in cats. The average time-action curve (Figure 3.1K) suggests that both analogs are relatively peakless. Considering the individual time action curves, however, it is clearly not safe to assume that either insulin detemir or insulin glargine are long-acting and peakless in any given patient (Figure 3.1A – 3.1J). Further investigation of the pharmacodynamic characteristics of both analogs is needed with emphasis on within-subject variability.

Translation of the results of clamp studies in healthy subjects to diabetic patients is limited by 3 factors. First, as discussed before, prolonged fasting increases insulin sensitivity even when glucose is infused, and therefore, clamp studies might overestimate the duration of action of the insulin studied. Second, a single-injection experiment might not reflect accurately the time-action curve of a long-acting insulin under steady-state conditions. This is true, however, only if the time interval between injections is short enough to allow overlap. Given the durations of insulin glargine and insulin detemir in our study, they are unlikely to have much overlap in most patients, even if administered twice daily. Third, clamp studies are usually performed on a relatively small number of subjects, and they might not reflect the full range of responses to the studied insulin in the target population. In people, it was found that even when

performed in diabetic patients, clamp studies do not fully reflect the potential range of clinical outcomes (Swinnen et al., 2008). For example, better glycemic control was achieved when insulin glargine was administered twice daily compared to once daily in type 1 diabetics despite its reported duration of action of over 24 hours. Insulin glargine was also associated with more hypoglycemic events than expected from its reported “flat” time-action curve (for further review see Swinnen et al., 2008). Because the clinical usefulness of each insulin is dependent upon its within-subject variability, large clinical trials in diabetic patients are necessary. In one small clinical study, once-a-day insulin glargine was comparable to twice-a-day NPH in controlling clinical signs and improving glycemic control in diabetic cats (Weaver et al., 2006). No data are available regarding clinical efficacy of insulin detemir.

In conclusion, insulin detemir and insulin glargine have shorter duration of action in cats than in people. Their time-action profiles seem less flat and more variable than in people and their peaks can be pronounced in some cats. In some cats, however, these insulin analogs might be useful as once-a-day drugs if their day-to-day variability is low. This, however, needs to be investigated further.

Footnotes

^a Purina DM, Nestlé Purina PetCare Company

^b Levemir®, Novo Nordisk Inc. Princeton, NJ USA

^c Lantus®, Sanofi-Aventis U.S. LLC, Bridgewater, NJ USA

^dV-Cath®, 3.0F, NeoMedical, Inc, Fremont, CA USA

^eBD Angiocath AutoGuard®, Becton Dickinson Infusion Therapy Systems, Inc., Sand, UT USA

^f50% Dextrose USP. Hospira Inc. Lakeforest, IL USA.

^g OneTouch Ultra, LifeScan Inc., Milpitas CA USA.

^h GraphPad Prism, GraphPad Software Inc., CA, USA.

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Tables and figures

Table 3.1 Summary statistics of insulin detemir and insulin glargine pharmacodynamic parameters.

		Detemir n = 10	Glargine n = 10	P value
Onset of action (hours)	mean \pm SD (95% CI)	1.8 \pm 0.8 (1.1 – 2.3)	1.3 \pm 0.5 (0.9 – 1.6)	0.03
	CV%	45.2%	38.6%	0.17
End of action (hours)	mean \pm SD (95% CI)	13.5 \pm 3.5 (11.0 – 16.0)	11.3 \pm 4.5 (8.0 – 14.5)	0.18
	CV%	26.4%	40.2%	0.48
Duration of action (hours)	mean \pm SD (95% CI)	11.7 \pm 3.6 (9.1 – 14.0)	10.0 \pm 4.7 (6.6 – 13.0)	0.31
	CV%	30.8%	47.0%	0.44

Table 3.1 (continued)

		Detemir n = 10	Glargine n = 10	P value
Peak action (mg/kg/min)	mean ± SD (95% CI)	5.3 ± 2.9 (3.2 – 7.4)	5.0 ± 2.3 (3.4 – 6.7)	0.7
	CV%	55.2	46.2%	0.5
Time to peak action (hours)	mean ± SD (95% CI)	6.9 ± 3.1 (4.7 – 9.2)	5.3 ± 3.8 (2.5 – 8.0)	0.31
	CV%	45.3%	72.9%	0.55
Total metabolic effect (g/kg)	mean ± SD (95% CI)	2.06 ± 1.2 (1.18 – 2.95)	1.67 ± 1.1 (0.9 – 2.5)	0.32
	CV%	59.81%,	66.9%	0.77

Figure 3.1 Insulin detemir (solid line) and insulin glargine (dashed line) time action curves. Panels A – J represent data from individual cats. The average curve is depicted in panel K.

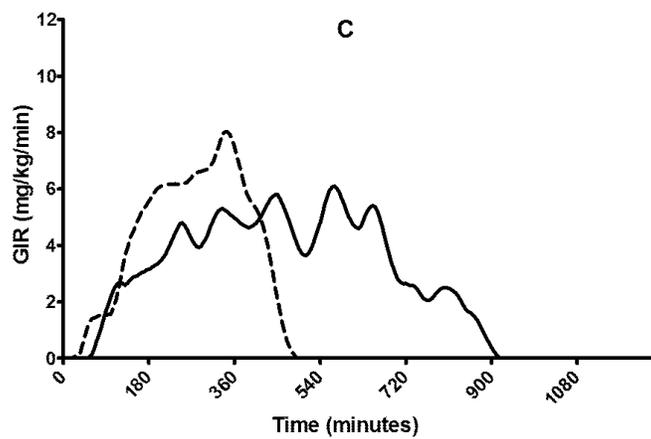
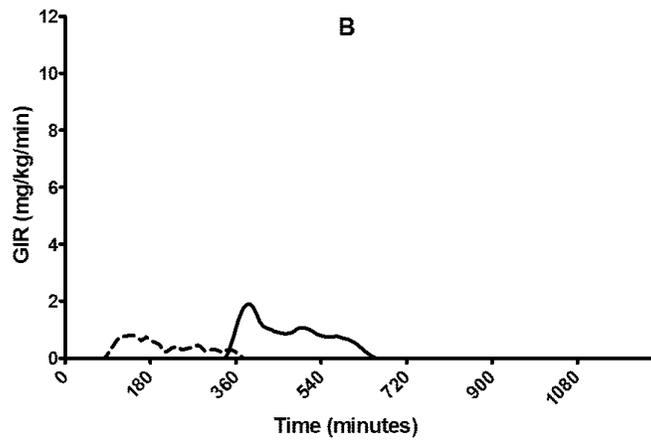
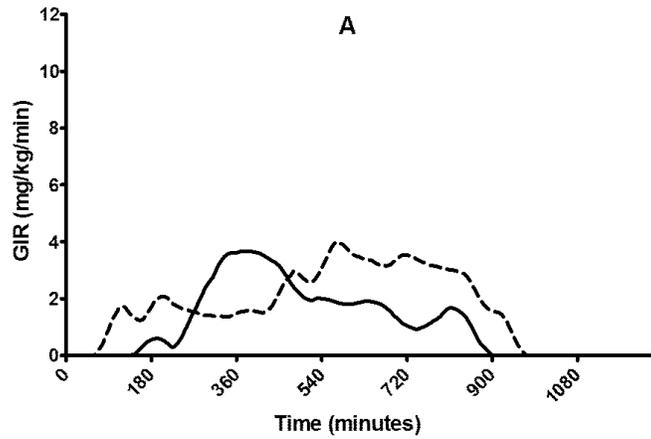


Figure 3.1 (continued).

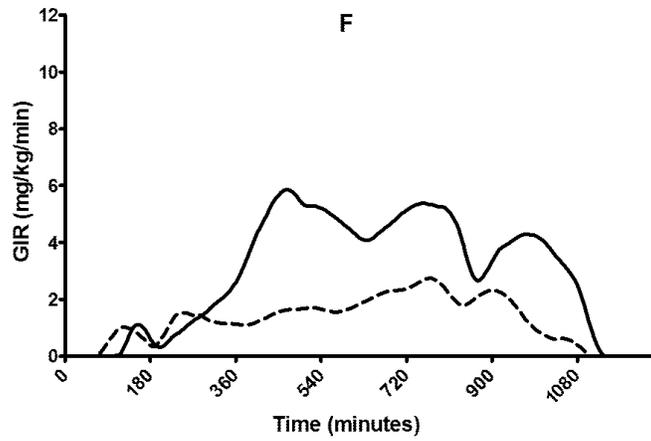
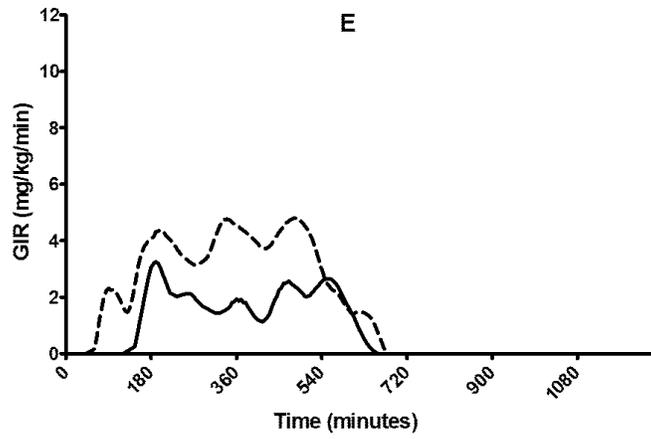
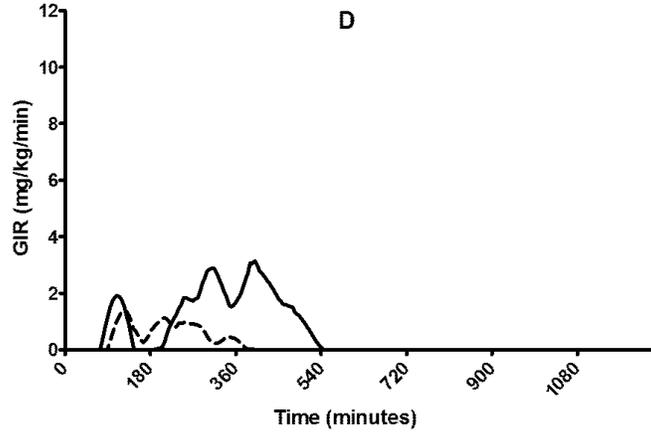


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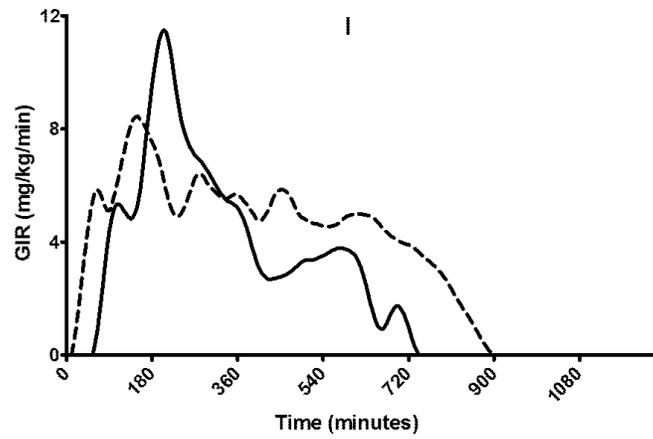
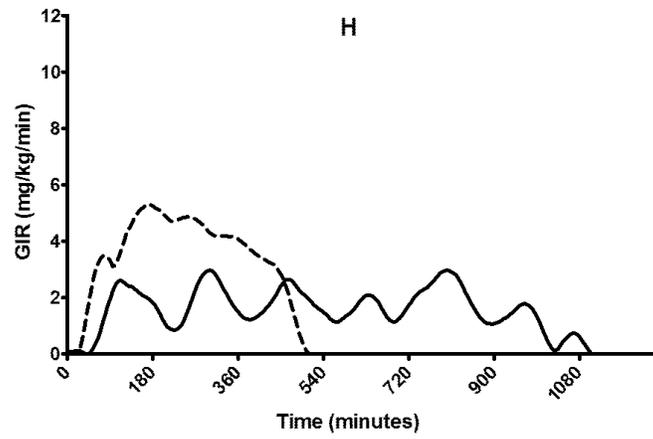
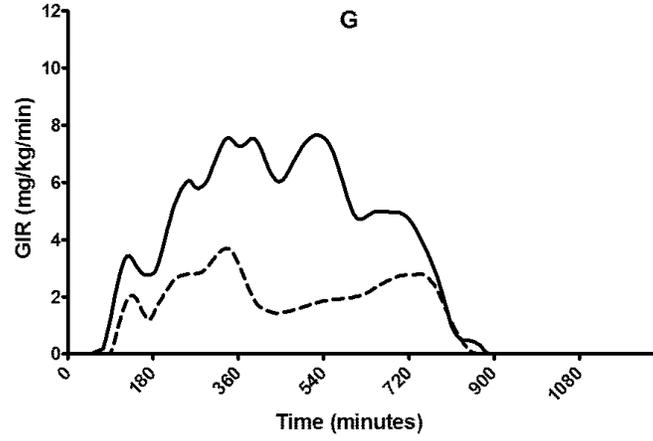
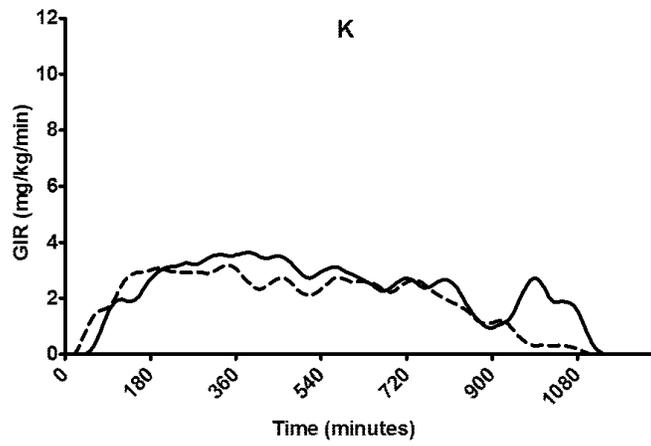
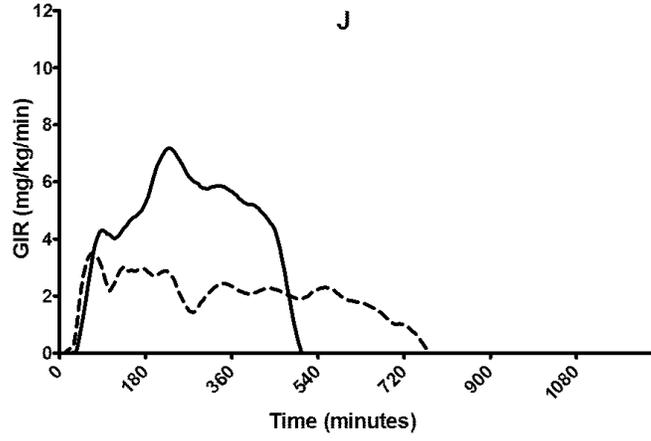


Figure 3.1 (continued).



CHAPTER 4

PHARMACOKINETICS OF INSULIN DETEMIR AND INSULIN GLARGINE IN HEALTHY CATS

Abstract

Background: The synthetic insulin analogs, insulin glargine and insulin detemir are comparable in their clinical use. Their chemical structure and hence their pharmacological properties are very different.

Objectives: To evaluate the pharmacokinetics of insulin detemir and insulin glargine in healthy cats.

Animals: Five young, healthy, neutered, purpose-bred cats

Methods: Randomized, cross-over design. Pharmacokinetics of insulin detemir and insulin glargine were determined during an isoglycemic clamp following a 0.5 U/kg subcutaneous injection. A sensitive insulin-analog ELISA was used to measure serum insulin.

Results: The pharmacokinetic profile of each analog correlated well with its pharmacodynamic profile. Insulin concentrations increased from pre-injection values within one hour in 9 of 10 experiments. Insulin concentrations returned to zero in the last measurement point in only one experiment. The difference in timing of last sampling was not significant but the insulin concentrations of the last sampling point tended to be higher in the insulin detemir experiments compared to the insulin glargine experiments.

Conclusion and clinical importance: With both analogs, insulin was still detectable in serum when the glucose-lowering effect (measured as an increase in glucose infusion rate during an isoglycemic clamp) was no longer detectable. Therefore, a threshold concentration for insulin action might be determined. This threshold could be used diagnostically to determine minimum frequency of insulin administration in diabetic cats.

Introduction

Studying the pharmacokinetics of an insulin formulation requires a sensitive and specific assay for the measurement of the insulin. The synthetic insulin analogs are based on human-recombinant insulin, and are altered biochemically to change their pharmacological properties. Amino-acid substitutions in the B26-B30 region alter the tendency of insulin to crystallize while retaining the ability to activate insulin receptors (Sheldon et al., 2009). Insulin analogs are mostly modified in this region. As a consequence of the alterations made in their sequences, insulin analogs are also immunologically different than native mammalian insulin. Thus, specific antibodies can be developed and used in antibody-based assays to differentiate native insulin from synthetic insulin. Feline insulin differs from human insulin in 4 amino acids, and from bovine insulin in only 1 amino acid (Hoenig et al., 2006). Thus, it is generally assumed that the immunogenicity of feline and bovine insulin is similar.

Insulin detemir has low affinity to the insulin receptor. Commercially, insulin detemir (Levemir®) is supplied in a molar concentration that is 4 times higher than the molar concentration of other insulin formulation so that the unit of activity per volume

would be comparable. Insulin detemir has a strong affinity to human albumin and it binds to it reversibly. Most circulating detemir is bound but only the un-bound fraction is free to cross endothelium and react with the insulin receptor. Thus, the pharmacokinetic data of insulin detemir cannot be directly compared with those of insulin glargine (or with the pharmacokinetic data of any other non-acylated insulin).

Materials and Methods

Blood samples for analysis of insulin analog concentration were collected in 5 of the 10 cats of the previously described experiment (Chapter 3). Samples were collected immediately prior to injection of insulin (time zero) and then every hour on the hour until the end of the isoglycemic clamp. The last sample was collected within half an hour of discontinuation of glucose infusion. Blood samples were collected through indwelling jugular catheters^a placed under sedation as described before. Blood samples for insulin measurement were collected at time zero and then every hour until the end of the isoglycemic clamp. Whole blood was collected in glass tubes and kept on ice a maximum of 12 hours. The blood was then centrifuged, and serum was harvested and frozen at -20° Celsius. Insulin concentrations were analyzed at the end of the study in one batch. Serum insulin detemir^b and serum insulin glargine^c concentrations were measured with the Iso-Insulin ELISA kit^d that was designed to react with recombinant insulin analogs. According to the manufacturer the assay is calibrated with human insulin (cross reactivity = 100%) and has a detection range of 3 – 100 mU/L with a sensitivity of 1 mU/L for human insulin. Cross reactivity is lower for insulin glargine (44%) and insulin detemir (22%). The lowest detectable concentrations of insulin glargine and insulin

detemir are ≥ 8 mU/L and ≥ 16 mU/L respectively. This cross reactivity for insulin analogs is much higher than in other insulin assays. For example, in an ultrasensitive insulin ELISA (from the same manufacturer), the cross reactivity with insulin glargine and insulin detemir is 9.9% and zero, respectively. Assay cross reactivity is reported by the manufacturer as 58% for bovine insulin which should be similar to feline insulin (so that the threshold for feline insulin should be $3/0.58$ mU/L = 5.2 mU/L, or 178 ng/L).

To validate the use of the Iso-Insulin assay for use in cats, fifteen feline serum samples were ran first in the Feline Insulin ELISA^e. These were banked samples that were collected in previous experiments after fasting and during various hyperglycemic conditions. These samples were free of any exogenous insulin. Insulin concentration in those samples ranged between 43.2 – 612.0 ng/L. A second set of aliquots of the same samples was then run in the Iso-Insulin ELISA. All samples were below detection range of the assay. In a separate study we measured fasting serum insulin with the Feline Insulin ELISA^e in the 5 cats of this study and in 5 others. The mean \pm SEM insulin concentration of the 5 cats of this study was 298.3 ± 22.5 ng/L in 25 measurements and for the 10 cats together it was 290.6 ± 20.9 ng/L in 45 measurements. These results indicated that fasted concentrations of feline insulin are unlikely to be detected by the Iso-Insulin kit.

For graphic representation, the serum insulin concentrations were adjusted based on the assay cross reactivity for the given insulin by dividing the measured concentration by a factor of 0.44 and 0.22 (the assay cross reactivity for insulin glargine and insulin detemir respectively). Concentrations that exceeded the assay range (100 mU/L) were designated a value of 100mU/L.

Statistical analysis was performed using computer software^f. Data are reported for the entire group (N = 5) as median (range). The Wilcoxon matched pairs test was used to compare medians between treatment groups. Statistical tests were performed as two-tailed tests and a P value < 0.05 was considered significant.

Results

Summary statistics of pharmacodynamic parameters were presented before in chapter 3, table 3.1. Adjusted insulin concentrations over time with their respective smoothed GIR over time in the 5 detemir clamps are presented in figures 4.1 F1-J1 (K1 is their mean). Adjusted insulin concentrations over time and smoothed GIR over time in the 5 glargine clamps are presented in figures 4.1 F2-J2 (K2 is their mean).

Pre-injection insulin concentrations in the Iso-Insulin assay were zero except on 2 occasions (unadjusted concentrations: 23.3 mU/L [figure 4.1 H1] and 20.5 mU/L [figure 4.1 J1]). Insulin concentrations increased from pre-injection values within one hour in 9 of 10 experiments. In the tenth it increased only in the second hour and this increase correlated with the increase in GIR. Visual inspection of general trends in figures 4.1 F-K reveals that changes in insulin concentrations correlated with changes in GIR.

Insulin concentrations returned to zero in the last measurement point in only one experiment. Excluding this one experiment, the last measured concentration in the 4 insulin glargine clamps (unadjusted to cross-reactivity) was (median, range) 5.7 (3.6 – 15.3) mU/L. The last measured concentration in the 5 insulin detemir clamps (unadjusted to cross-reactivity) was (median, range) 19.3 (18.5 – 24.3) mU/L. In the 5 cats, the median difference between the 2 clamps in the last insulin concentrations measurement

was 17.4 mU/L (3.2 – 20.7). The difference was not significant but with obvious trend towards higher concentrations in the insulin detemir clamps compared to the insulin glargine clamps (P = 0.0625). The difference in timing of last sampling was not significant (30 minutes [-240 – 640 minutes], P = 1.0).

Discussion

In this study we found good agreement between pharmacokinetic and pharmacodynamic data of each of the insulin analogs. An increase in concentration of insulin was detected within one hour in all clamps except for one glargine clamp in which it was detected after 2 hours and correlated with the increase in GIR. Shapes of insulin curves resembled the GIR curves. This is despite the fact that on many occasions the concentrations exceeded the assay range. Because sampling was limited to once every hour, assessing time of appearance and duration of stay is inaccurate.

It is unclear why 2 of the pre-injection samples had a false positive reading of insulin detemir. We have shown that serum samples from fasted cats as well as serum samples from glucose-stimulated cats do not normally contain sufficient concentrations of feline insulin to cross react with the Iso-Insulin ELISA. It is possible that the fasted concentrations of insulin in these 2 cats were high enough to exceed the threshold of the Iso-Insulin assay for feline insulin. In a separate study (incretin study, chapters 5 and 6), fasted serum samples from these 2 cats were tested on multiple occasions and the results were variable but not high enough to be detected in the Iso-Insulin assay (median 204 ng/L, [range 151-428] in one cat and 346 ng/L, [range 309-383] in the other). It is also possible that the false positive reading was caused by some non-specific matrix

interference but this was not repeatable in these 2 cats. Ideally, insulin concentrations should have been analyzed in all samples using the Feline Insulin ELISA in parallel to the Iso-Insulin assay. These might have explained the aberrant reading in the 2 cats in time zero and also shed light on the results of the last measurement in each clamp. Insulin concentration did not decrease to zero at the end of the clamp in 9 of 10 experiments. This could be because the endogenous insulin was high enough to be measured by the ELISA.

Alternatively, it is possible that the low insulin concentrations at the end of each clamp represent mostly exogenous insulin and that these low concentration resulted in metabolic activity that was low enough to allow a decrease in GIR to zero without appreciable decrease in BG. This second alternative seems more likely because of three reasons. First, the isoglycemic clamps were performed at sub-euglycemic blood glucose concentrations to avoid artificial prolongation of duration of action (discussed previously in Chapter 3). In this scenario, it is possible that the remaining circulating insulin at the end of the clamp was not enough to lower the BG below our chosen threshold but was still present and may even had some metabolic activity. Second, the unadjusted insulin concentration tended to be higher at the end of the detemir clamps compared to the end concentration at the end of the glargine clamp. This difference is most likely related to a difference between treatments because all other factors were controlled as this was a repeated measures experiment. The different treatments could have led to different metabolic effects and to a change in insulin sensitivity for example, that might be reflected by a difference in endogenous insulin once the experiment is over. This is unlikely, however, because the end of action was not significantly different between the

2 clamps in each cat. Third, endogenous insulin is expected to be low at the end of the clamp because of prolonged fasting. As mentioned before, the mean fasting endogenous insulin concentrations in these 5 cats in a different study was 298.3 ± 22.5 ng/L. Even if all the insulin that was measured at the end of each clamp was endogenous insulin and if feline insulin has across-reactivity similar to bovine insulin in this assay then the measured concentrations at the end of these detemir clamps represent a value of 619 – 838 ng/L. This is very unlikely to be the concentration of endogenous insulin in these 5 cats after over 24 hours of fasting (prolonged fasting increases insulin sensitivity and decreases endogenous insulin secretion).

If the measured insulin at the end of each clamp represents the remaining exogenous insulin and not endogenous insulin, insulin detemir at an adjusted concentration of 84 – 110 mU/L (2900 – 3800 ng/L) was associated with a return of GIR to zero. This might be important clinically. If measured after an over night fast, one single measurement of serum insulin concentration in the Iso-Insulin ELISA can indicate whether insulin detemir is still present in concentrations that should have a glucose lowering effect and give an indication of its duration of action. If the concentration is lower than 110 mU/L it is unlikely to still have a metabolic effect. It is important to note that insulin concentrations in this range were measured in cats after a meal rich in protein and fat, although insulin was measured in a different assays (Appleton et al., 2001; Mori et al., 2009). In other studies we have stimulated insulin secretion in healthy cats with IV glucose, oral glucose, lipids or amino acids. Insulin concentrations were measured with Feline Insulin ELISA and rarely exceeded 60 mU/L (2000 ng/L) except after oral administration of amino acids. It should be even less likely to measure such high

concentrations of insulin in a diabetic cat, unless it has circulating exogenous insulin. It is important to remember that such high concentrations of insulin detemir are necessary for a metabolic effect because of its low affinity to the insulin receptor and because it is mostly albumin-bound and not available for action.

The Iso-Insulin ELISA is calibrated with human insulin standards and its results are presented as units of activity per liter. This means that for detemir the results should be divided by an unknown factor because the affinity of insulin detemir for the feline insulin receptor is unknown.

Footnotes

^a V-Cath®, 3.0F, NeoMedical, Inc, Fremont, CA USA.

^b Levemir®, Novo Nordisk Inc. Princeton, NJ USA.

^c Lantus®, Sanofi-Aventis U.S. LLC, Bridgewater, NJ USA.

^d Iso-Insulin ELISA, Mercodia AB, Uppsala, Sweden

^e Feline Insulin ELISA, Mercodia AB, Uppsala, Sweden

^h GraphPad Prism, GraphPad Software Inc., CA, USA.

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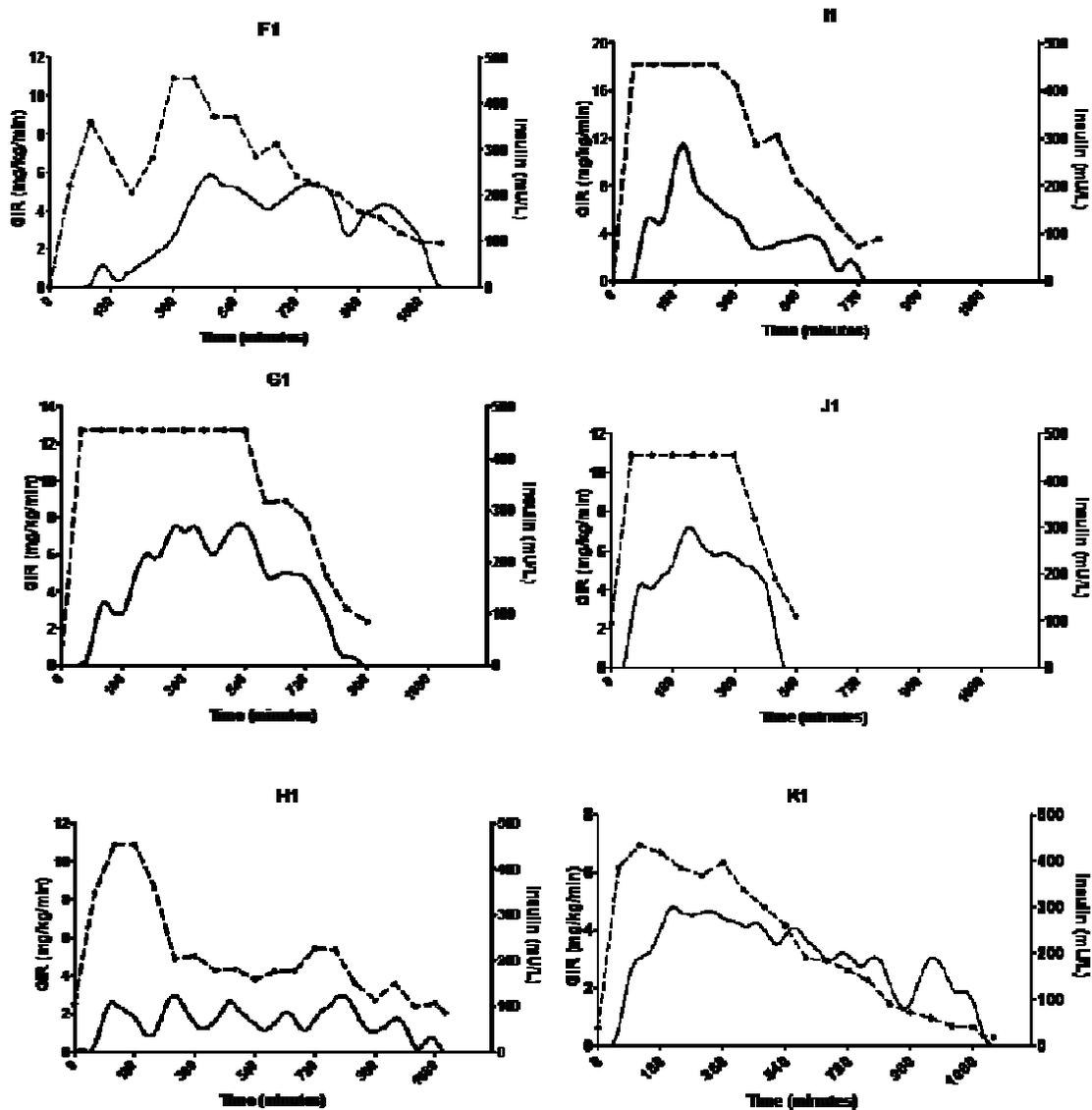
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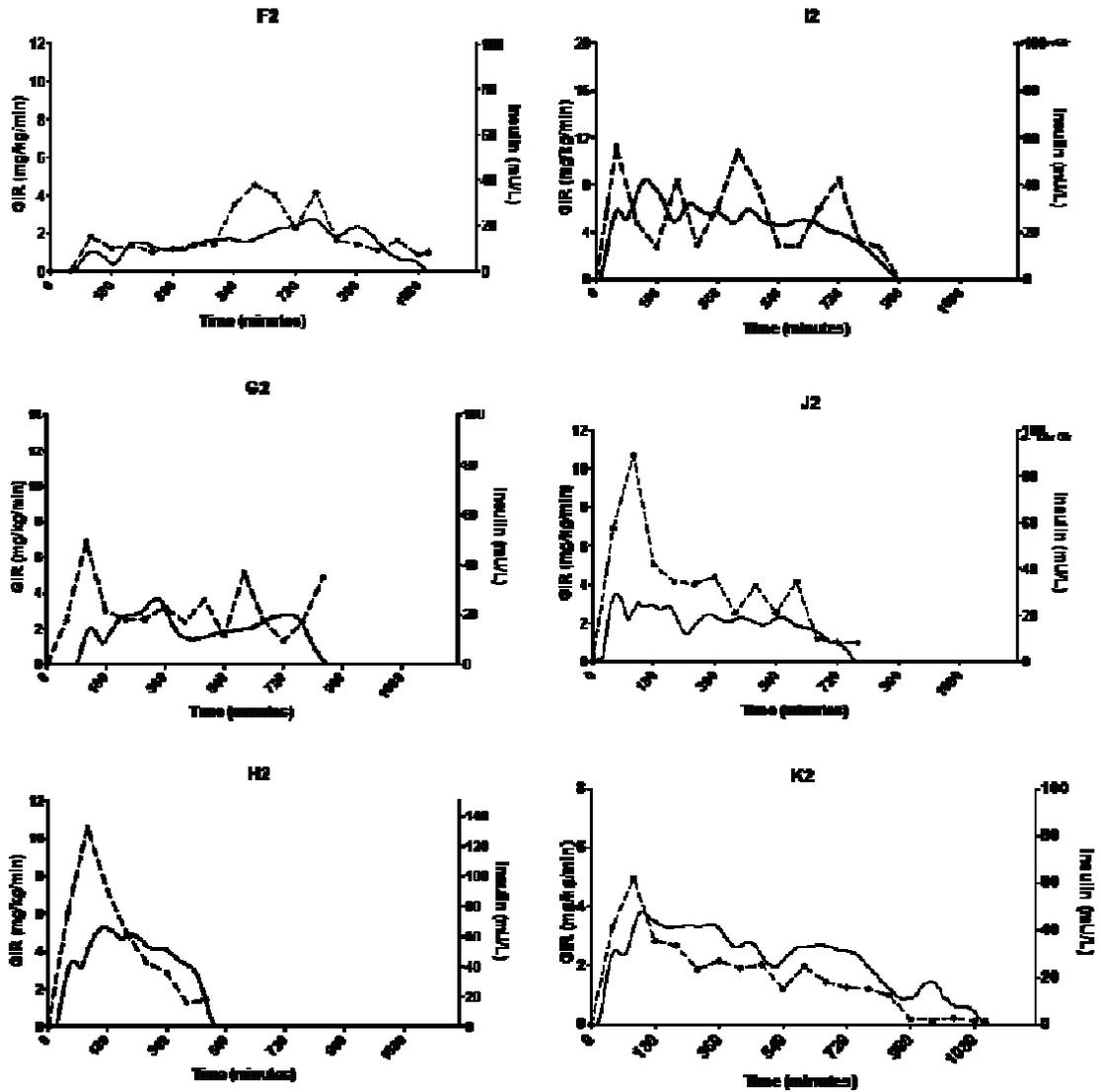
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Figures

Figures 4.1 GIR (solid line) and insulin concentration (dashed line) during insulin detemir isoglycemic clamps (F1 – K1) and insulin glargine isoglycemic clamps (F2-K2). Panels F1 – J1 and F2 – J2 represent data from individual cats and correspond to panels F – J in figure 3.1. The average curves for the 5 cats are depicted in panels K1 and K2.



Figures 4.1 (continued).



CHAPTER 5

THE INCRETIN EFFECT IN CATS:

COMPARISON BETWEEN ORAL GLUCOSE, LIPIDS, AND AMINO ACIDS

Abstract

Background: Naturally occurring type 2 diabetes mellitus (DM) is common in domestic cats, and is a compelling model for the disease in people. Little is known about incretin hormones in cats.

Objectives: To study the incretin effect in cats and compare the effect of oral glucose, lipids or amino acids on serum concentrations of insulin, total GIP and total GLP-1.

Methods: Randomized, cross-over design. Ten healthy cats. Glucose, lipid or mixed amino acids were administered through nasoesophageal tubes on separate study days. Blood glucose concentrations (BG) were matched between experiments by measuring BG every 5 minutes and infusing glucose intravenously at a changing rate (isoglycemic clamp). Intravenous glucose infusion (IV glucose) with no prior treatment served as control. Insulin concentrations were measured with a feline insulin ELISA. Total GIP and total GLP-1 concentrations were measured with human ELISA's that were validated for use in cats. The incretin effect was estimated as the difference in insulin area under the curve (AUC) after oral compared to IV glucose. Total glucose infused (TGI), and area under the insulin, GIP and GLP-1 curves were compared between different treatments.

Results: On average, 0.49 g/kg of glucose administered IV resulted in slightly higher BG compared to 1 g/kg oral glucose. Insulin concentrations were not significantly different. BG and TGI were not significantly different in the three oral challenges. Insulin AUC after amino acids was 5.46 ± 1.33 fold higher than insulin AUC after lipids and 2.2 ± 0.3 fold higher than insulin AUC after oral glucose. Total GIP concentrations increased significantly after lipids (32 fold, range 10 – 137) and amino acids (12 fold, range 2 – 35) but not after oral glucose. Total GLP-1 concentrations increased significantly after all 3 oral stimulations to a similar degree (after glucose 1.6 fold [1.2 – 2.9], lipids 1.5 fold [1.2 – 3.9], and amino acids 1.7 fold [1.4 – 2.5]).

Conclusion and clinical importance: Potentiation of insulin secretion after oral ingestion of glucose is minimal in cats and is not mediated by GIP. Lipids are more potent GIP secretagogues compared to amino acids, but both cause rapid and pronounced increases in serum GIP concentrations. GLP-1 secretion is stimulated equally by glucose, lipids or amino acids.

Introduction

Stimulation of insulin secretion by glucose ingestion is partly due to direct action of glucose on the pancreatic beta cells. In addition, the entero-insular axis enhances the total amount of insulin released. The incretin effect is the difference between insulin secretion after glucose ingestion and that after an isoglycemic intravenous glucose infusion (Nauck et al., 1986). In people, the incretin effect accounts for about 80% of insulin secreted after glucose ingestion (Nauck et al., 1986). Incretin hormones potentiate insulin secretion, resulting in increased insulin secretion at any given concentration of

blood glucose. Incretins also participate in regulation of pancreatic beta-cell differentiation, proliferation and survival; they affect glucagon secretion, slow the rate of gastric emptying and increase satiety. Thus, incretins play a major role in glucose homeostasis (Kim and Egan, 2008).

The domestic cat is a compelling animal model of diabetes because it spontaneously develops a form of the disease that closely resembles human type 2 diabetes. Cats, humans and macaques are the only species that spontaneously develop islet amyloid deposits (Henson and O'Brien, 2006). Other similarities include onset at middle age or older, a genetic predisposition, association with obesity as a major risk factor, and impaired beta cell function with residual but declining insulin secretion. In contrast to other models of type 2 diabetes, cats are obligate carnivores. As such, their natural diet is based mostly on fat and protein and contains less carbohydrate. Sugar-sensing in taste buds is therefore unnecessary to the cat, and indeed cats lack the T1R2 sweet-taste receptor (Li et al., 2006). Sugar-sensing, however, is also an important function of enteroendocrine cells that secrete incretin hormones. Glucose is sensed in enteroendocrine cells by T1R2 sweet-taste receptors, but also by mechanisms similar to its sensing in pancreatic beta cells (involving its entry through SGLT1/GLUT-2, its metabolism through glucokinase and then closure of K_{ATP} channels) (Jang et al, 2007; Kim and Egan, 2008; Reimann et al., 2008; Parker et al., 2009). Because cats lack the T1R2 sweet-taste receptor, and because they are obligate carnivores, it is likely that their enteroendocrine response to glucose and other nutrients would be different than in other species.

Two hormones account fully for the incretin effect in people: Glucose-dependent Insulinotropic Peptide (GIP) and Glucagon-Like Peptide -1 (GLP-1). The degree of GIP and GLP-1 stimulation by different nutrients is species-dependent. Fat is more potent than carbohydrates in stimulating GIP secretion in people but the opposite is true in pigs. Amino acids are weak stimulators of GIP secretion in people but strong stimulators of it in dogs and rats (Deacon, 2005; Kim and Egan, 2008). GLP-1 secretion is stimulated by ingested lipid, carbohydrate and protein as well as by GIP (Damholt et al., 1998; Gunnarsson et al., 2006; Kim and Egan, 2008). Different distributions of GLP-1-secreting cells (L cells and K/L cells) along the intestinal tract in different species determine, in part, the importance of different stimuli of GLP-1 secretion (Damholt et al., 1999; Kim and Egan, 2008).

Based on the view of the domestic cat as an obligate carnivore, we hypothesized that the glucose-induced incretin effect in cats would be less than is observed in other species. We also explored the differential effects of glucose, lipid and amino acids on secretion of incretin hormones.

Materials and Methods

Animals: Ten young, healthy, purpose-bred cats were used in this study (4 spayed females and 6 neutered males, median age 48 months [range 36 – 58 months], mean BW was 4.8 ± 0.68 kg, with no significant difference between males and females). Cats were group-housed in AAALAC-accredited facilities and all animal use was approved by the University of Illinois Institutional Animal Care and Use Committee. All cats were acclimatized and socialized for at least 4 weeks prior to the start of experiments, and

extensive environmental enrichment was provided. Cats were fed commercial cat food^a and monitored daily by physical exams. Body weight was measured weekly. Routine laboratory tests (including CBC, serum chemistry panels, total T4, coagulation panels and urinalysis) were performed at first arrival and just prior to the first part of the experiment in each cat. PCV, total solids and CBC's were repeated prior to each part of the experiment in each cat.

Study design: A repeated measures study design was used. The experiment was divided to 4 parts: Oral glucose tolerance test (OGTT), isoglycemic clamp (IGC), lipid challenge with an isoglycemic clamp (LIGC) and amino acids challenge with an isoglycemic clamp (AAIGC). First, an OGTT was performed and blood glucose concentrations (BG) were measured every 5 minutes until euglycemia was restored. In the second part, IGC was performed 2-3 days after the OGTT. During the IGC, BG was measured every 5 minutes and glucose was infused IV at a changing rate. The rate of infusion was adjusted to match or exceed the BG that was recorded during the OGTT at the next time point by no more than 10%. The third and fourth parts of the experiment were done four weeks later: LIGC and AAIGC were performed 3 days apart, in random order. In these clamps the target BG was 0-10% less than the BG recorded during the IGC.

On the day of each experiment, BG was measured twice between 8 and 9 a.m. If hyperglycemia was detected, the experiment was postponed until the next day. At 9 a.m. a sample was obtained for hormone measurement and then a solution of glucose^b (2 ml/kg), lipids^c (1.7 ml/kg) or amino acids^d (8.5 ml/kg) was administered through a nasoesophageal tube. The 3 solutions were calorically equivalent (3.4 Kcal/kg) and the

volumes were matched by flushing the tubes with water to reach a total volume of 9 ml/kg. All 3 solutions (water flush included) were administered over 4 minutes. Time zero was defined as the end of glucose and amino acid administration in the OGTT and AAIGC respectively, and the beginning of glucose infusion in all 3 glucose clamps (IGC, LIGC and AAIGC). In the LIGC, the end of lipid administration was defined as -15 minutes; BG was still measured every 5 minutes and time zero was still defined as the beginning of the IGC. For glucose intravenous infusion, a 50% glucose solution was diluted with saline to a 20% solution. Infusion rate was set on a syringe pump.

Serum samples for insulin, GIP and GLP-1 measurement were collected during the OGTT, LIGC and AAIGC at baseline (immediately prior to nutrient administration or at time zero in the IGC) and then at 15, 30, 45, 60, 75, 90 and 120 minutes and then every 30 minutes thereafter until euglycemia was restored. In the IGC, insulin was measured similarly but GIP and GLP-1 were measured only at baseline and at 30 minutes and only in 5 cats. Blood was collected through indwelling jugular catheters^e. The samples were collected into chilled glass tubes and then immediately centrifuged (at 4°C and 4000 RPM) and separated. Serum was stored at -20°C until analysis.

Catheters and nasoesophageal tube placement: On the morning before each part of the experiment, cats were sedated with intramuscular injections of dexmedetomidine (0.009 mg/kg) and butorphanol (0.22 mg/kg) to facilitate catheter placement. Atropine was given (0.022mg/kg intramuscularly) to prevent bradycardia. Jugular catheters^e were placed prior to the OGTT. These catheters were maintained by flushing heparinized saline daily until the end of the study. Cephalic catheters^f were placed prior to each IGC and were removed at the end of the IGC. These were used exclusively for glucose

infusion. The sedation was reversed with atipemazole (0.009 mg/kg) and the cats were monitored until full recovery. Food was given as usual at 3 pm, and one hour later the cats were lightly sedated with acepromazine (0.04 mg/kg) and butorphanol (0.22 mg/kg) and a 3.5F red rubber nasoesophageal feeding tube was placed. The tube was pre-measured and marked to reach the ninth rib. Cats were conscious during the procedure and proper placing was verified by observing an intact swallowing reflex, and by checking the tube for negative pressure after placement. Elizabethan collars were used to prevent the cats from removing the nasoesophageal tubes. Food and water were then withheld until after the experiment the next day. Nasoesophageal tubes were placed prior to OGTT, AAIGC and LIGC but not prior to IGC.

Glucose and hormone measurements: Blood glucose concentrations were measured with a hand-held point-of-care glucose meter^g that was validated for use in cats (Gilor et al., 2010). Insulin concentrations were measured with a feline ELISA^h. Total GIP concentrations and total GLP-1 concentrations were measured with human ELISA kits^{i,j} that were validated for use in cats. Feline GIP and feline GLP-1 were not available and therefore spiking and recovery were not performed. In the GIP ELISA the intra-assay CV was $4.9 \pm 3.3\%$ and the inter-assay CV was $6.2 \pm 2.1\%$. Linear regression for expected vs. observed concentrations of GIP in serial dilutions are presented in figure 5.1. The results of the regression were: $R^2 = 0.99$ ($P < 0.0001$), Slope = 0.98 ± 0.02 , Y intercept = 3.67 ± 2.25 . In the GLP -1 ELISA the intra-assay CV was $7.3 \pm 5.6\%$. Linear regression for expected vs. observed concentrations of GLP-1 in serial dilutions are presented in figure 5.2. The results of the regression were: $R^2 = 0.85$ ($P < 0.0001$), slope = 0.60 ± 0.06 , Y intercept = 0.58 ± 0.38

Statistical analysis: Statistical analysis was performed using computer software^k. The Shapiro-Wilk test was used to assess deviance from normal distribution of data. Data are reported as mean \pm SE if normally distributed or as median (range). Comparisons between treatment groups were analyzed with paired or repeated measures tests. OGTT and IGC were performed in 10 cats. LIGC and AAIGC were performed in only 8 out of the 10 due to sampling catheter failure. In repeated measures tests that included OGTT and/or IGC, only cats with a full set of data were included. Paired t-tests or one-way repeated measures ANOVA were used for normally distributed data. The Wilcoxon signed-rank test and the Friedman's test were used for data that were not normally distributed. Fold increase or fold difference were calculated as the deviation of a ratio from 1.0 using a one sample t test or the Wilcoxon signed-rank test. Comparisons between time points were analyzed with one-way repeated measures ANOVA.

The area under the curve (AUC) was calculated using the trapezoidal method for the BG and for hormones concentrations. AUC was used to represent total hormone secretion.

All statistical tests were performed as two-tailed tests and a P value < 0.05 was considered significant.

Results

Baseline BG's in the IGC experiment were lower than in the other experiments but the differences were not significant (IGC: 81.8 ± 2.8 mg/dl vs. 87.5 ± 2.3 in the OGTT, 87.5 ± 3.4 in the LIGC and 84.8 ± 1.4 in the AAIGC).

Glucose-induced incretin effect: The AUC of blood glucose concentrations in the IGC was slightly higher ($6.7 \pm 2.8\%$) than the AUC in the OGTT ($P = 0.038$, Fig. 5.3). Compared to the 1 g/kg of glucose that was given orally for the OGTT, the total glucose infused during the IGC was significantly lower at 0.49 ± 0.06 g/kg ($P < 0.0001$, Fig. 5.4). Total insulin secretion was not significantly different ($P = 0.19$, Fig. 5.5) between the OGTT ($46772 \text{ ng} \cdot \text{L}^{-1} \cdot \text{min}$ [19815 – 304406]) and the IGC ($52168 \text{ ng} \cdot \text{L}^{-1} \cdot \text{min}$ [19146 – 79448]) with a median fold difference of 23% (-74 – 670).

Median baseline GIP concentrations (of all experiments) were 11.4 pg/ml (3.5 – 61.1). GIP concentration did not increase significantly during the OGTT. Median baseline GLP-1 concentrations (of all experiments) were 8.9 pmol/L (4.5 – 26.5). GLP-1 concentrations peaked 30 minutes after glucose administration (fold increase 1.5 [0.9 – 2.8]) and returned to baseline at 45 and 120 minutes (Fig. 5.6). It reached a maximum fold increase from baseline of 1.6 (1.2 – 2.9). Time to maximum was 75 minutes (30 – 150).

GIP and GLP-1 concentrations did not change significantly between time zero and 30 minutes of the IGC.

Lipid and amino acid stimulation: The AUC of blood glucose concentrations in the LIGC was lower than the AUC in the IGC ($-9 \pm 4\%$) but the difference was not significant ($P = 0.15$, Fig. 5.7). The AUC of blood glucose concentrations in the AAIGC was significantly lower than the AUC in the IGC ($-10 \pm 3 \%$, $P = 0.002$, Fig. 5.8). The AUC of blood glucose concentrations in the LIGC and AAIGC was not significantly different than the AUC in the OGTT ($1 \pm 5.5 \%$, $P = 0.87$ and $1 \pm 6.4\%$, $P = 0.89$ respectively) or from one to the other ($2 \pm 6\%$, $P = 0.78$). The total glucose infused

during the LIGC or AAIGC was not significantly different than 1 g/kg (LIGC: 0.91 ± 0.14 g/kg, $P = 0.53$; AAIGC: 0.88 ± 0.09 g/kg, $P = 0.25$, Fig. 5.4). Insulin concentration increased within 15 minutes of lipid administration (at time zero of the isoglycemic clamp, while BG was still unchanged). Insulin AUC during AAIGC was significantly higher than in the LIGC (fold difference $120 \pm 30\%$, $P = 0.004$). Insulin AUC during LIGC was significantly higher than in the OGTT (fold difference $160 \pm 60\%$, $P = 0.008$), Fig. 5.9).

GIP concentration peaked 15 minutes after lipid administration (24 fold increase [range 10 – 75 fold], Fig. 5.10) and remained elevated after 135 minutes (120 minutes of the glucose clamp). It reached a maximum fold increase of 32 (10 – 137) from baseline. Time to maximum was 52.5 minutes (15 – 105). GIP concentration peaked 15 minutes after amino acid administration (fold increase 11 [1 – 28]) and remained elevated after 120 minutes. It reached a maximum fold increase of 12 (2 – 35) from baseline. Time to maximum was 52.5 minutes (15 – 90). Total GIP secretion (as measured by the area under the time-concentration curve) in response to lipids was 2.8 fold (1.5 – 9.0) greater than to amino acids ($P = 0.008$) but individual patterns of GIP serum concentration curves after lipids or amino acids stimulations were remarkably similar in 5 out of 8 cats (Figs 5.11a – h).

GLP-1 concentrations during LIGC and AAIGC were measured in only 6 cats because of insufficient sample volume. GLP-1 peaked 15 minutes after lipid administration (fold increase 1.5 [1.2 – 3.9], Fig. 5.12) and returned to baseline at 30 minutes. Time to maximum was 30 minutes (15 – 75). GLP-1 concentration peaked 15 minutes after amino acid administration (fold increase 1.7 [1.4 – 2.5]) and returned to

baseline at 45 minutes. It reached a maximum fold increase from baseline of 2.6 (1.5 – 9.3). Time to maximum was 30 minutes (15 – 60). Total GLP-1 secretion (as measured by the area under the time-concentration curve) was not significantly different between glucose, lipids or amino acids oral stimulations ($P = 0.56$).

Discussion

In this study we examined the incretin effect in response to glucose stimulation in healthy cats. Consistent with the fact that the cat is an obligate carnivore, we found that the incretin effect in cats is minimal when compared to other species. The lack of significant difference between insulin AUC during the OGTT and IGC might suggest that in cats there is no incretin effect in response to glucose, however, our findings mostly suggest the opposite.

First, it should be considered that the lack of difference in insulin AUC in itself signifies an incretin effect given the higher AUC of BG concentration during IGC compared with the OGTT, combined with the difference between the total amount of glucose infused vs. glucose administered orally. If there was no incretin effect, one would expect that the excess of oral glucose (twice as much as the IV glucose) would result in a higher BG concentration. The opposite occurred here. If there was no incretin effect, one would also expect a higher BG during the IGC to result in a higher AUC of insulin. The opposite occurred here. The combination of these 2 significant differences with the lack of difference in insulin AUC's, supports the presence of an incretin effect, albeit small.

Second, it is possible that the incretin effect in cats is mostly mediated by a decrease in glucagon (resulting in a decrease in hepatic glucose output) and less by an increase in insulin itself. This is supported by the results of a recent study in which the effect of a DPP-4 inhibitor in cats on lowering BG were mostly mediated by a decrease in glucagon and less so by an increase in insulin (Furrer et al., 2010). This is also consistent with our finding that GLP-1 secretion was stimulated by oral glucose but GIP stimulation was not. In other species, both incretin hormones contribute an insulinotropic effect after glucose ingestion, but they have opposite effects on glucagon secretion: GLP-1 decreases glucagon secretion while GIP increases it (Kim and Egan, 2008; Carr et al., 2008). Lack of GIP secretion in response to oral glucose will lead to an overall decrease in insulinotropic effect but at the same time a net decrease in glucagon secretion. This should result in a decreased hepatic glucose output and a greater tolerance towards oral glucose.

Third, it should be considered that the lack of a significant difference in insulin AUC was a result of lack of statistical power caused by the large variation in our insulin results. At the same time, it is possible that a lack of statistical power precluded us from finding the opposite result: That the insulin concentrations were in fact higher during the IGC, as expected if there is no incretin effect and the BG during the IGC is higher than the in the OGTT. In that case, the large difference in total glucose infused vs. oral glucose still requires an explanation. If there is no incretin effect, the similarity in BG in the OGTT and the IGC can only be explained by a lack of absorption of a significant fraction of the orally administered glucose. In a recent study, OGTT was performed in cats but with a dose of 2 g/kg of glucose (Hoenig et al., 2010). With that dose, 7/21 cats

developed signs of gastrointestinal upset (vomiting or diarrhea) that might be caused by the osmotic pool of unabsorbed glucose. Interestingly, the BG during OGTT in that study resulted in similar or lower BG than in our study giving twice the amount of glucose we gave. This difference is either a result of a more pronounced incretin effect (as seen in people with increasing doses of oral glucose; Nauck et al., 1986), a lack of complete glucose absorption in both studies, or simply due to differences in methods. In contrast to our study, Hoenig et al. (2010) administered glucose to cats under sedation. It is possible that the sedatives affected GI motility and thus changing the glucose excursion. One limitation of our study was that glucose and other nutrients were administered via a nasoesophageal tube. The tube was placed the day before each experiment and by the time the experiment was started there was no apparent discomfort to the cats (except for occasional sneezing). Also, our cats were very well-acclimated to the conditions of the laboratory and had participated in previous studies. Although we did not perceive the cats to be stressed, it cannot be completely ruled out that some degree of stress was affecting them. In that case, a sympathetic response could inhibit insulin secretion, increase glucagon secretion and interfere with the incretin effect. The small difference in baseline BG between the OGTT and the IGC might be evidence of stress in these cats. Perhaps if stress had been completely avoided, a greater incretin effect would have been seen. A stress-free voluntary consumption of a glucose solution is not a viable option in cats, however, and we consider the method of glucose administration we chose to be the most practical.

If there is a glucose-induced incretin effect in cats, it might be mediated by GLP-1 but it is clearly not mediated by GIP. GIP, however, may be responsible for an incretin

effect stimulated by other nutrients. Insulin concentration increased despite euglycemia 15 minutes after lipid administration. This was accompanied by a significant and marked increase in GIP concentration. Interestingly, in people, oral triglycerides trigger GIP release, but this results in insulin secretion only during hyperglycemia (Ross and Dupre, 1978). We did not measure triglyceride and fatty acid concentrations in the blood and it is possible that the increase in insulin concentration was a result of direct stimulation of pancreatic beta cells by triglycerides or fatty acids. It is unlikely, however, that within 15 minutes of administration, lipids would be digested, absorbed into the lymphatic system, and enter the bloodstream in high enough concentrations to cause the stimulation of insulin secretion we observed. It is more likely that there was a direct stimulation of GIP and GLP-1 secretion which resulted in potentiation of insulin secretion.

In dogs, secretion of GLP-1 is not directly stimulated by oral glucose. Rather, K cells are stimulated by glucose and L cells are stimulated by GIP, probably in a paracrine way (Damholt et al., 1999). Although dogs are the closest model to cats (at least among animal models in which the incretin effect has been reported), it seems that in stimulation of L cells by glucose, cats are more similar to other species. Stimulation of GLP-1 secretion was observed in our study with no appreciable increase in serum GIP concentrations. Although this does not rule out a paracrine effect of GIP on L cells, it makes it unlikely unless glucose stimulated GIP secretion very weakly or GIP did not enter the systemic circulation.

In this study, caloric and volume equivalent stimulations with oral glucose, lipids or amino acids were followed by similar responses in GLP-1 secretion. In contrast, glucose did not stimulate GIP secretion, amino acids were associated with a strong

stimulation of GIP secretion, and lipids with an even stronger stimulation. AUC's of BG after glucose, lipid and AA stimulations were not significantly different. Therefore, the difference in insulin secretion between the 3 stimulations cannot be accounted for by differences in blood glucose. Lipids and amino acids can directly stimulate insulin secretion and, therefore, the difference in insulin concentrations between the 3 experiments cannot be assumed to be an incretin effect. The causal association with rapid and remarkable increase in GIP and GLP-1 concentrations and a similar increase in insulin concentrations, together with the known effect of GLP-1 and GIP in other species, support the notion that incretins have an insulintropic effect in cats.

As for the magnitude of stimulation by the 3 nutrients, it is important to note that the 3 solutions we administered were not equal in molar concentrations. We chose to equate the caloric content of the 3 solutions because that would give more clinically useful information regarding the relative benefit of a given nutrient. K and L cells are stimulated by fatty acids via specific G protein-coupled receptors (GPR). The mechanism of stimulation by amino acids and glucose are more controversial but it is either mediated by GPR's, by sodium-coupled transporters, or by both. Regardless, stimulation of K and L cells by nutrients is proportional to the molar content of the nutrients. Notably, degradation of triglycerides to fatty acids is critical to stimulation of GLP-1 secretion (Beglinger et al., 2010) so that the final molar content of triglycerids affecting stimulation is 3 times the original molar content. In our study the molar content of amino acids and glucose were 5.6 and 3.8 times higher than the molar content of fatty acids (assuming all lipids were digested to fatty acids). We can therefore hypothesize that, on a molar basis, lipids are more potent stimulators of GLP-1 secretion in cats

compared to amino acids and glucose, even though on a caloric basis they resulted in similar responses. Moreover, degree of stimulation of K and L cells varies between saturated and unsaturated fatty acids and between different amino acids. For example, in people GLP-1 secretion is stimulated more by monounsaturated fatty acids than polyunsaturated fatty acids or saturated fatty acids (Beysen et al., 2002). In cell cultures, glutamine is a stronger stimulus of GLP-1 secretion than asparagine and alanine (Reimann et al., 2004). In fact, on a molar basis, glutamine and glucose stimulate GLP-1 secretion to a similar degree (Greenfield et al., 2009). The lipid solution we used was composed mainly of unsaturated fatty acids. As obligate carnivores, the cat's natural diet contains mostly saturated fat and it is plausible that their L and K cells respond to saturated fat more than to unsaturated fat. Further studies of the differential effect of specific fatty acids and amino acids should elucidate the potential of manipulating the composition of the diet to affect incretin secretion in cats. This might have implications to the treatment of diabetes and obesity.

In people basal GIP concentrations in peripheral venous blood are typically 9-11 pM and they increase 5-10 fold post-prandially. Basal GLP-1 concentrations in peripheral venous blood of people are typically 5-10 pM and increase 2-3 fold after a meal (Kim and Egan, 2008). In cats, we found baseline GIP concentrations that are about a fifth of the basal concentration in people, but there was much higher increase in response to lipids. Basal GLP-1 concentrations are similar in cats and people, with similar post-prandial fold increases. The differences in basal GIP between people and cats could be explained by a low cross reactivity of the anti-GIP antibody used in our assay. In contrast to GLP-1, the homology of GIP among mammals is not 100%. We

used a human GIP ELISA because feline GIP or feline specific GIP assays are not commercially available. If low cross reactivity was the issue, then post-stimulation concentrations of GIP were also underestimated. This would strengthen the finding that GIP is not stimulated by glucose in cats and that lipid- or amino acid-stimulated concentrations are much higher than in people. In people, GIP is crucial in maintaining glucose tolerance in the post-prandial phase. Lack of GIP response to glucose and an overall weak incretin effect could render the cat relatively glucose-intolerant, and might lead to inappropriate glycemic control in cats fed a diet high in carbohydrates. A recent study, however, showed no correlation between dietary carbohydrate content and development of diabetes in cats (Slingerland et al., 2009).

Conclusion: In this study we found that the glucose-dependent insulinotropic effect of incretins in cats is minimal when compared to other species. We also explored the differential effects of glucose, lipids and amino acids on secretion of incretin hormones. In contrast to other species, glucose did not stimulate GIP secretion. Oral lipids and amino acids were followed by a fast and pronounced increase in serum GIP concentrations, with much greater fold increase than reported in other species. GLP-1 responses were similar to previous reports from other species.

Footnotes

^a Purina DM, Nestlé Purina PetCare Company

^b Dextrose 50% USP. Hospira Inc. Lakeforest, IL USA

^c Liposyn II 20% Hospira Inc. Lakeforest, IL USA

^d Aminosyn II 10% Hospira Inc. Lakeforest, IL USA

^e BD Angiocath AutoGuard®, Becton Dickinson Infusion Therapy Systems, Inc., Sand, UT USA

^f V-Cath®, 3.0F, NeoMedical, Inc, Fremont, CA USA

^g OneTouch Ultra, LifeScan Inc., Milpitas CA USA

^h Feline Insulin ELISA, Mercodia AB, Uppsala, Sweden

ⁱ Human GIP (total) ELISA, Millipore Inc., St. Charles, MO, USA

^j GLP-1 (7-36 and 9-36) ELISA , ALPCO Diagnostics, Salem, NH, USA

^k GraphPad Prism, GraphPad Software Inc., CA, USA

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Figures

Figure 5.1 Expected vs. observed concentrations of GIP in serial dilutions. $R^2 = 0.99$ ($P < 0.0001$).

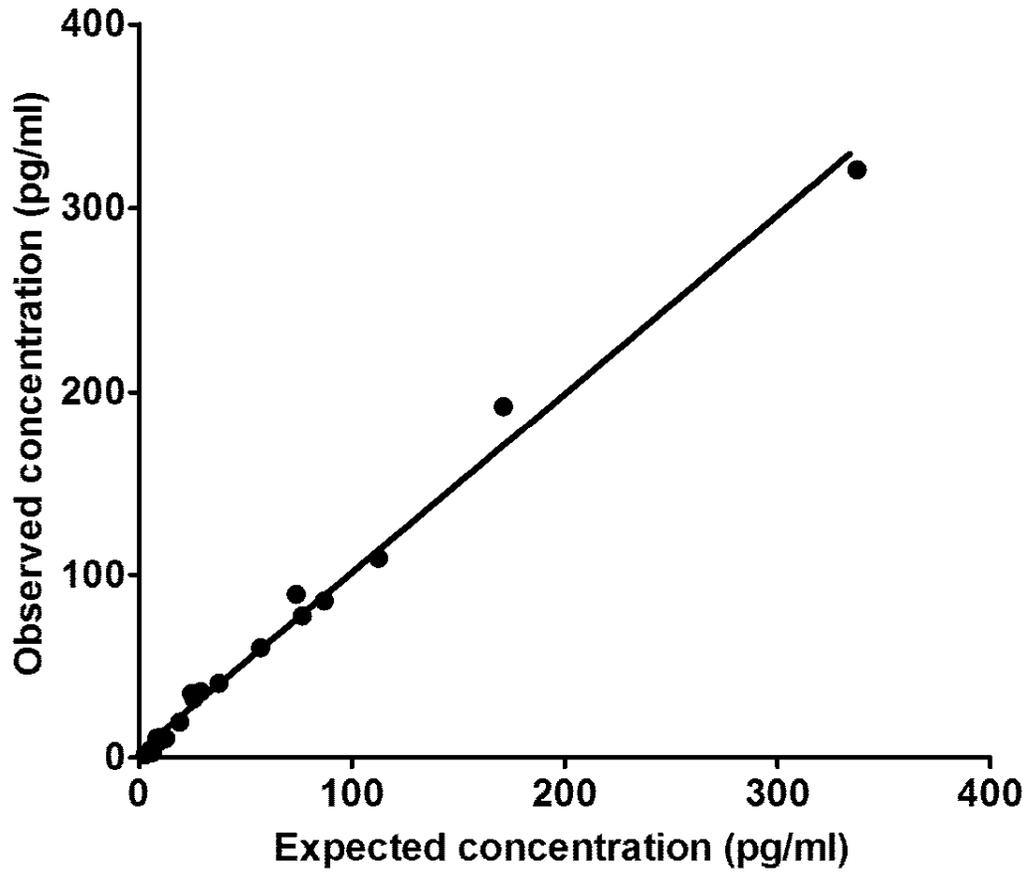


Figure 5.2 Expected vs. observed concentrations of GLP-1 in serial dilutions. $R^2 = 0.85$
($P < 0.0001$).

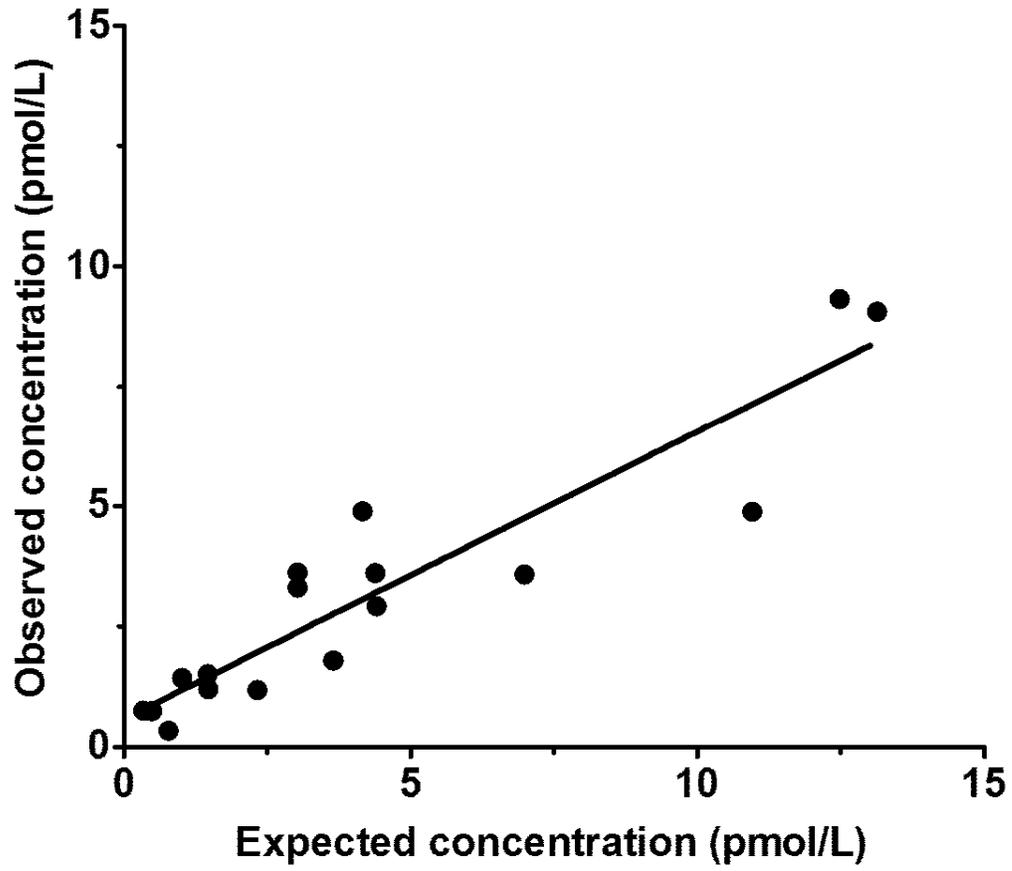


Figure 5.3 Blood glucose concentrations during OGTT (solid line) and IGC (broken line). Error bars represent the standard error of the mean.

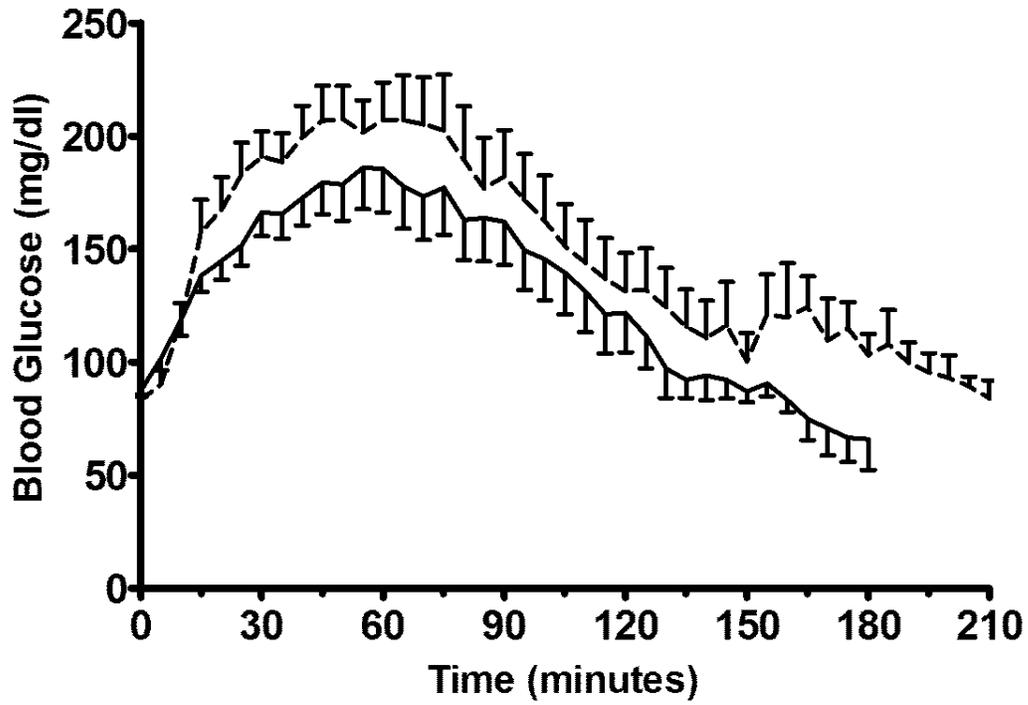


Figure 5.4 Box plot of the total glucose infused during OGTT, IGC, LIGC and AAIGC.

The boxes represent the interquartile intervals from the 25th to the 75th percentiles. The solid horizontal bars through the boxes represent the medians, and the minimum and maximum are represented by the capped vertical bars. * indicates statistically significant difference as compared to all other groups.

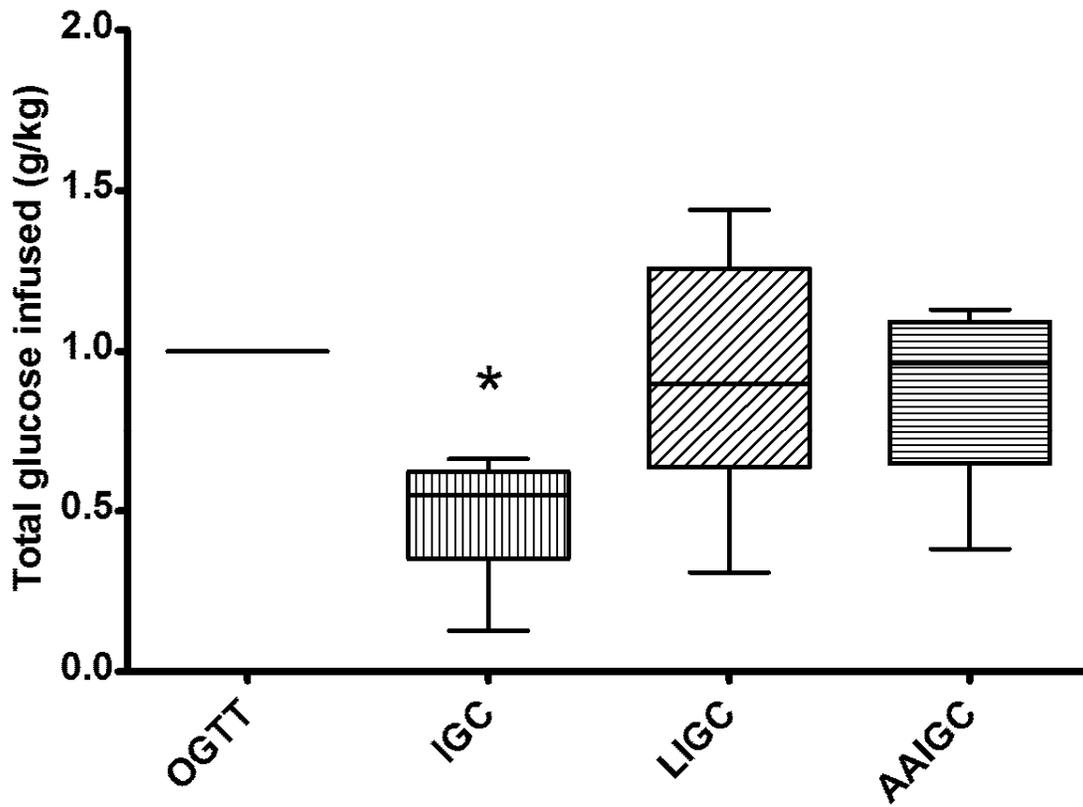


Figure 5.5 Serum insulin concentrations (ng/L) during OGTT (solid line) and IGC (broken line). Error bars represent the standard error of the mean.

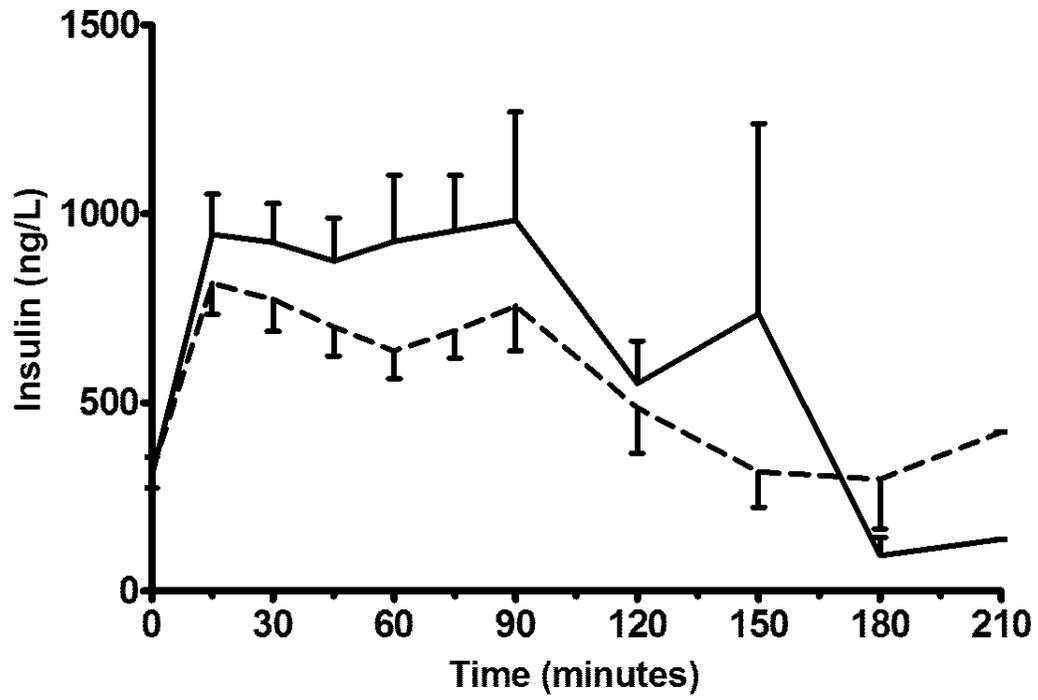


Figure 5.6 Serum total GLP-1 concentrations (pmol/L) during OGTT. Error bars represent the standard error of the mean. * indicates statistically significant difference from baseline.

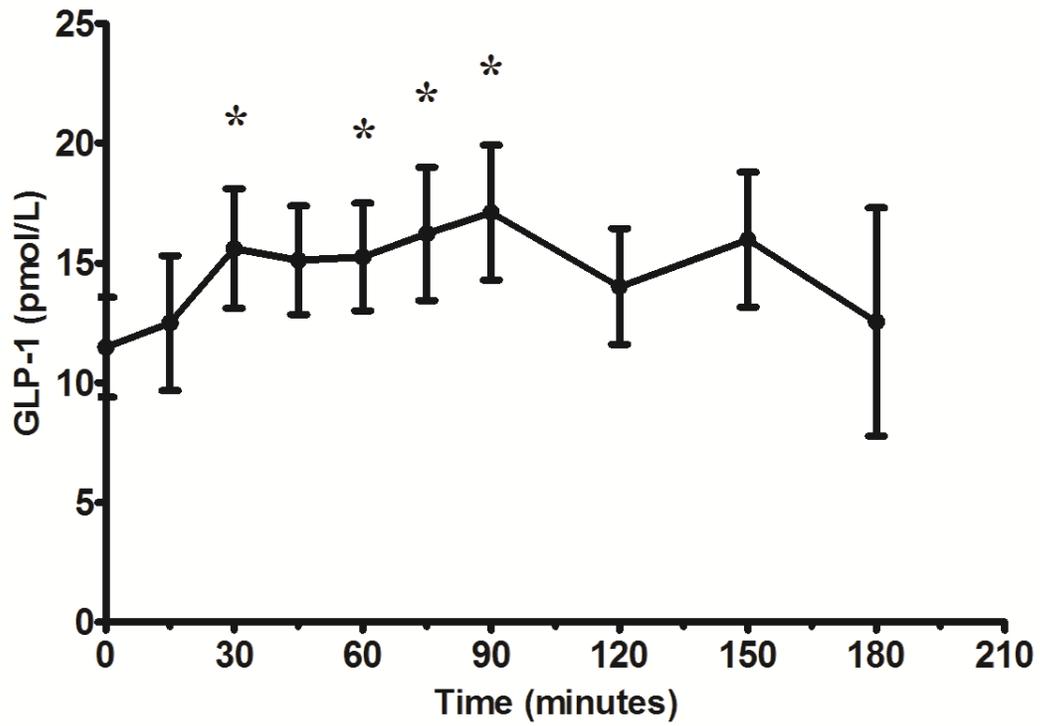


Figure 5.7 Blood glucose concentrations during LIGC (solid line) and IGC (broken line). Error bars represent the standard error of the mean.

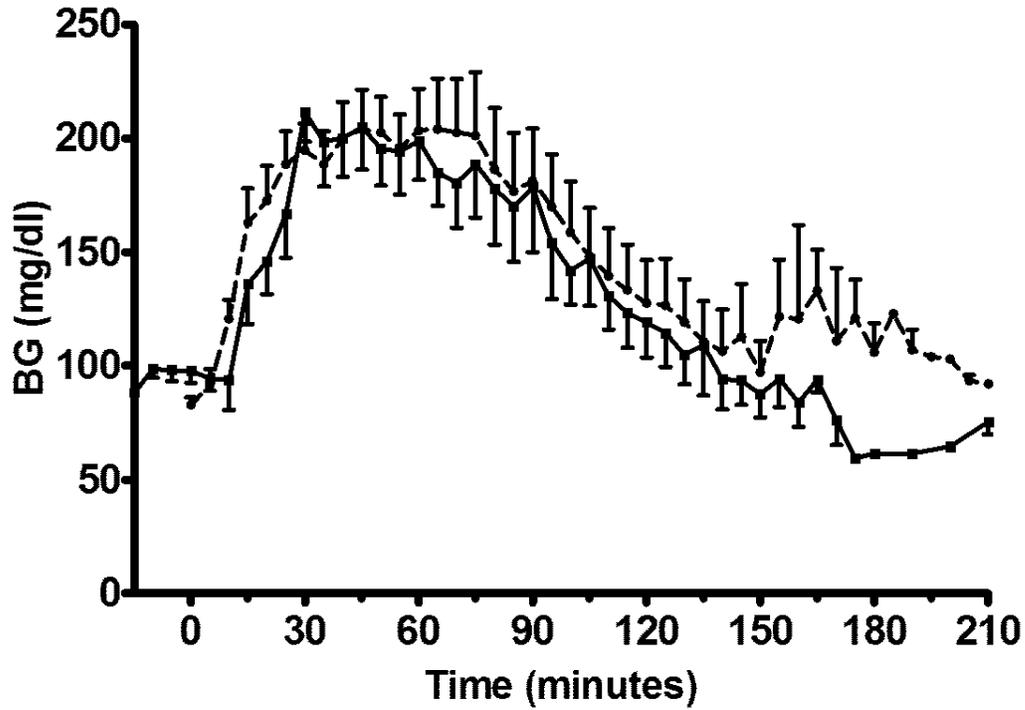


Figure 5.8 Blood glucose concentrations during AAIGC (solid line) and IGC (broken line). Error bars represent the standard error of the mean.

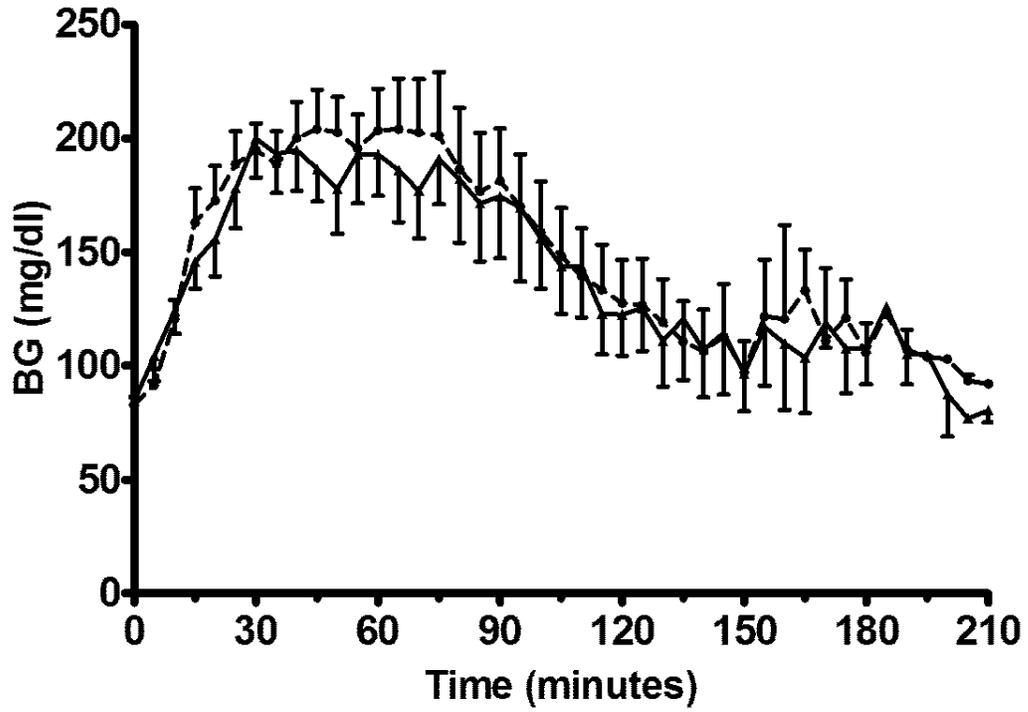


Figure 5.9 Serum insulin concentrations (ng/L) during OGTT (solid line), LIGC (dashed line) and AAIGC (dotted line). Error bars represent the standard error of the mean.

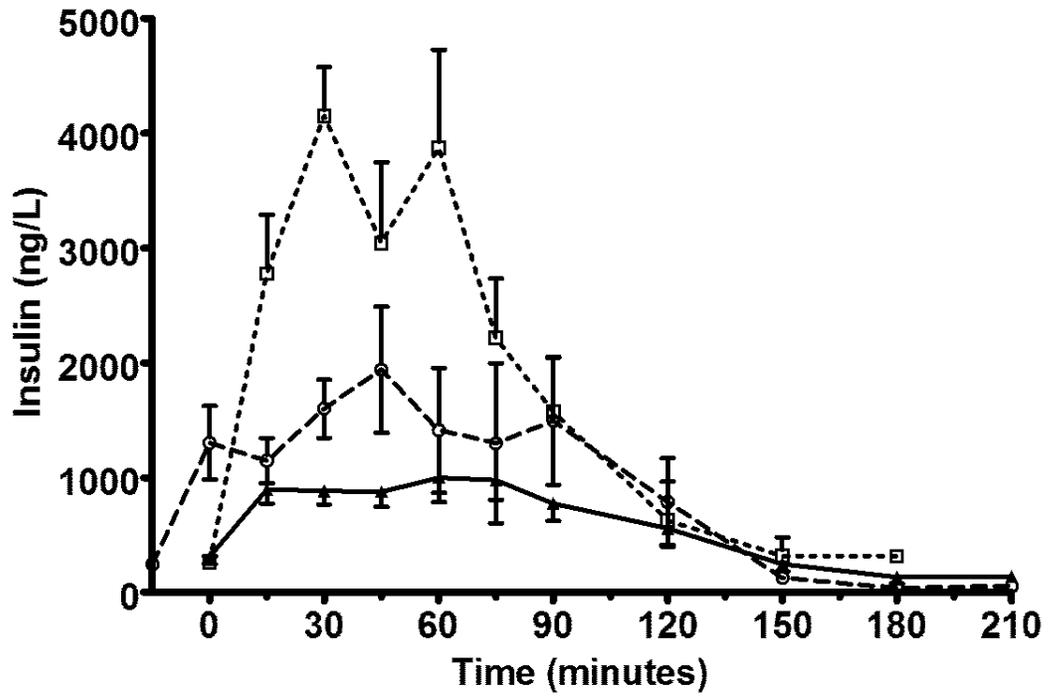


Figure 5.10 Serum total GIP concentrations (pg/ml) during OGTT (solid line), LIGC (dashed line) and AAIGC (dotted line). Error bars represent the standard error of the mean.

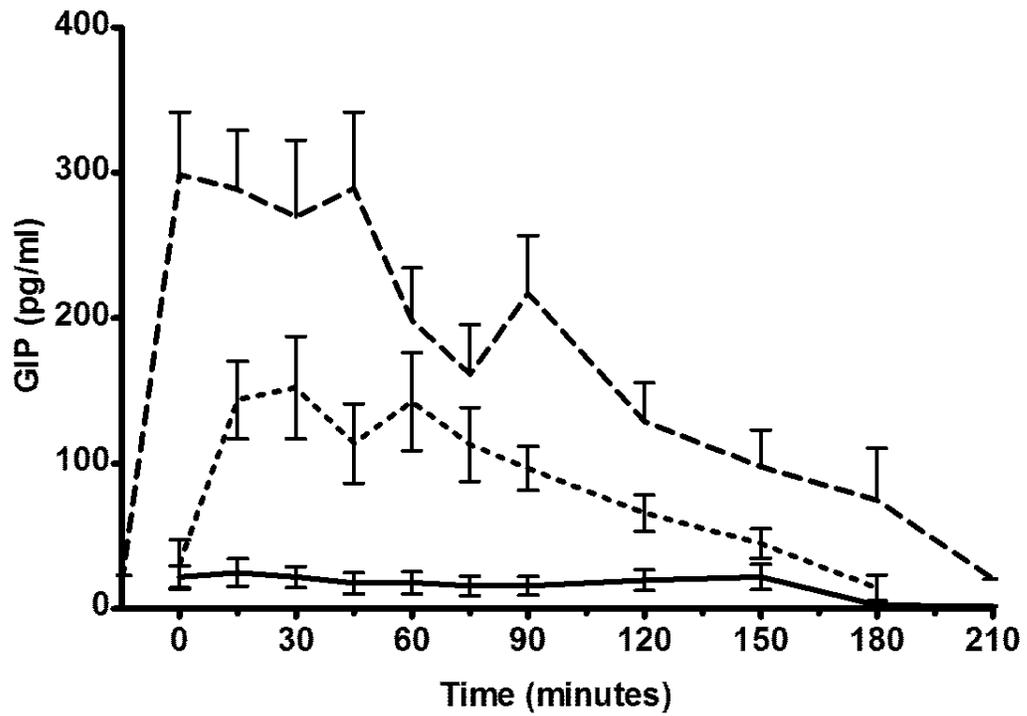


Figure 5.11 A-H Serum total GIP concentrations (pg/ml) during LIGC (dashed line), and AAIGC (dotted line) in 8 individual cats.

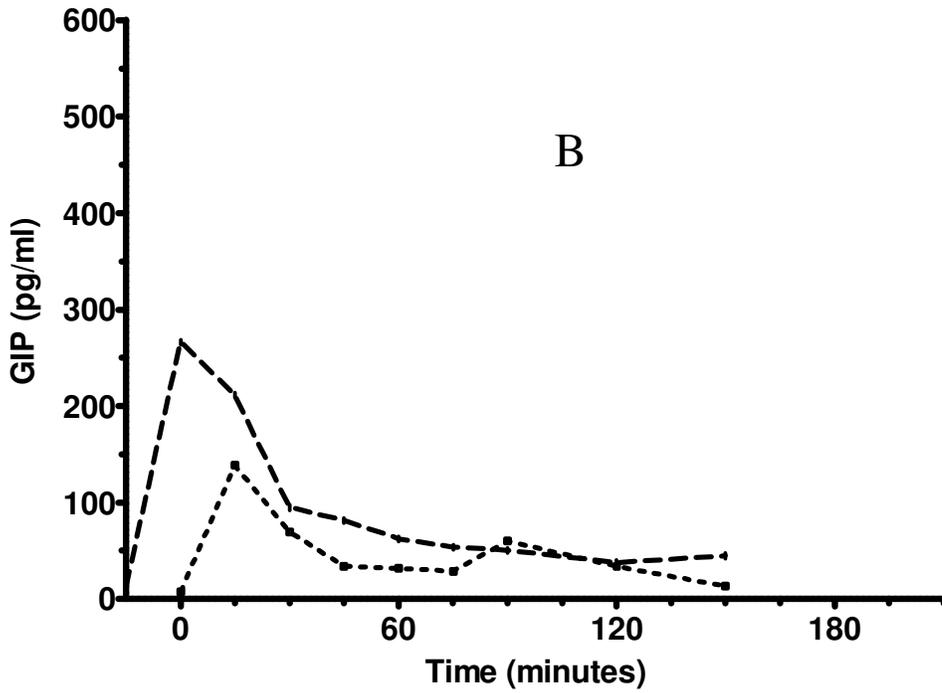
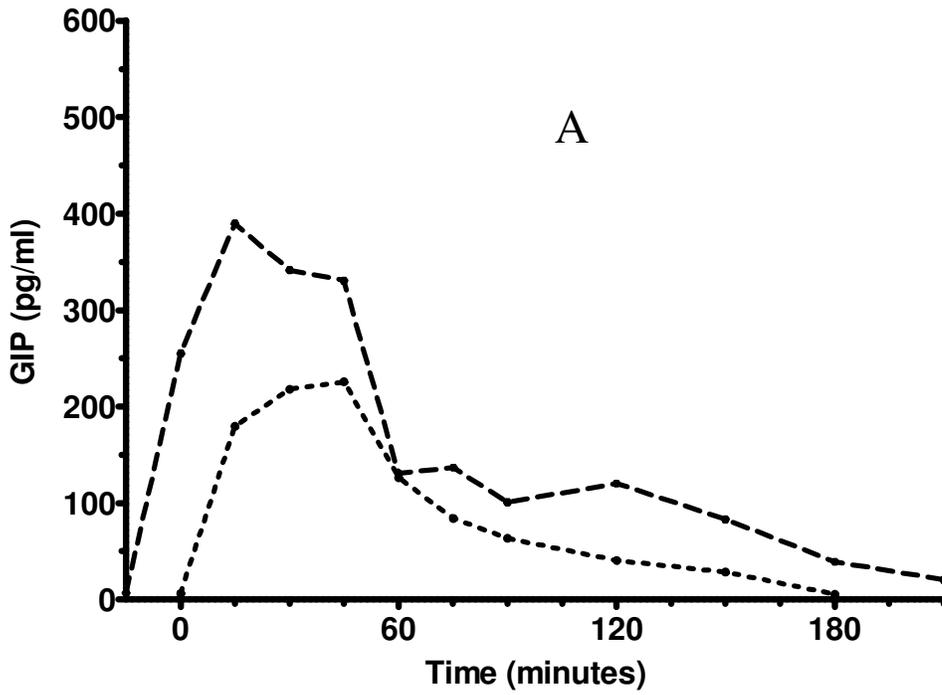


Figure 5.11 (continued)

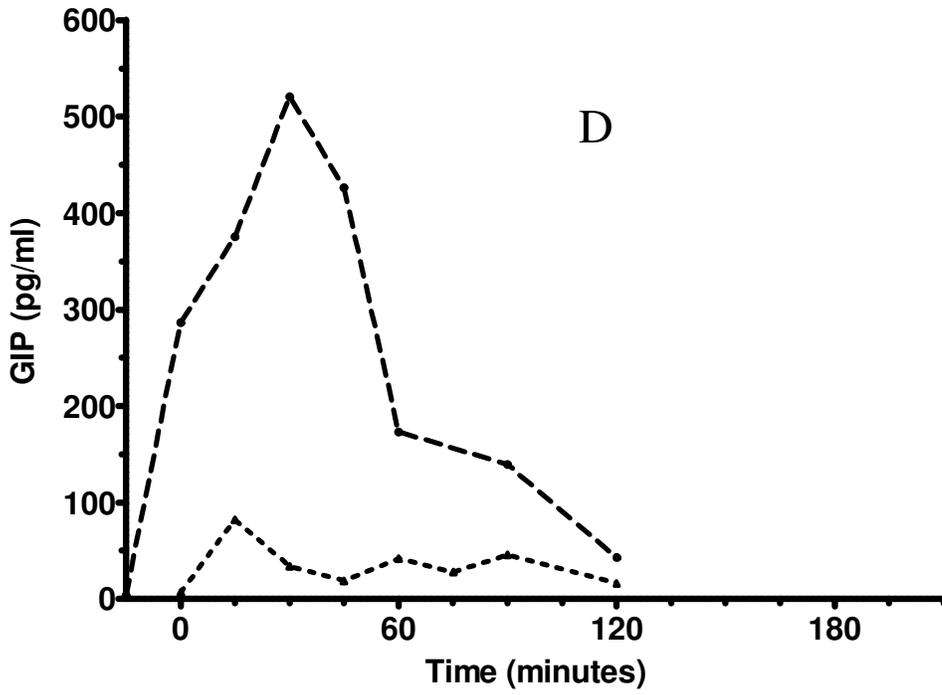
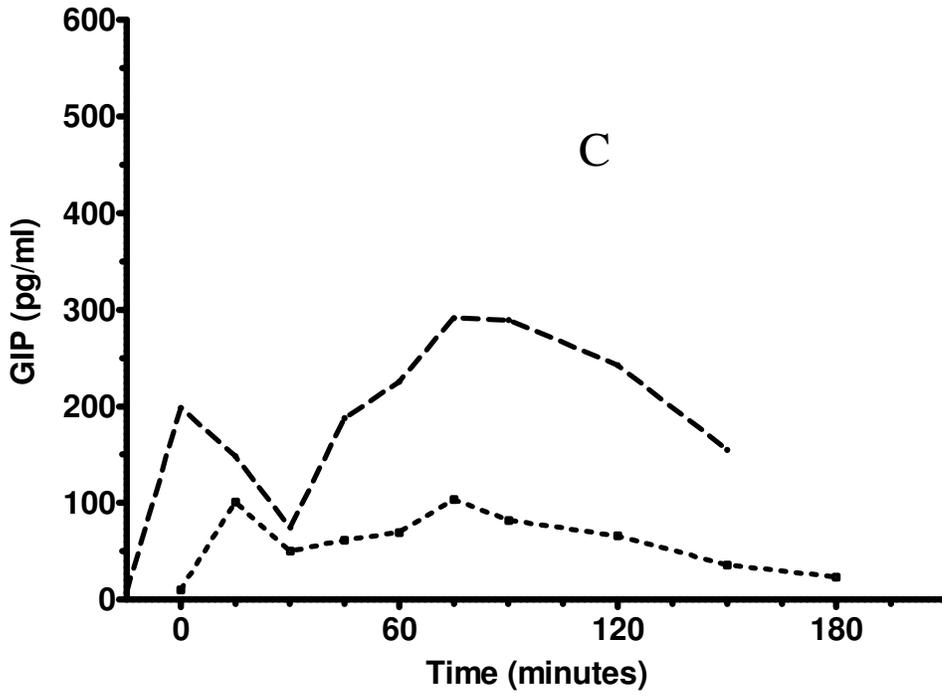


Figure 5.11 (continued)

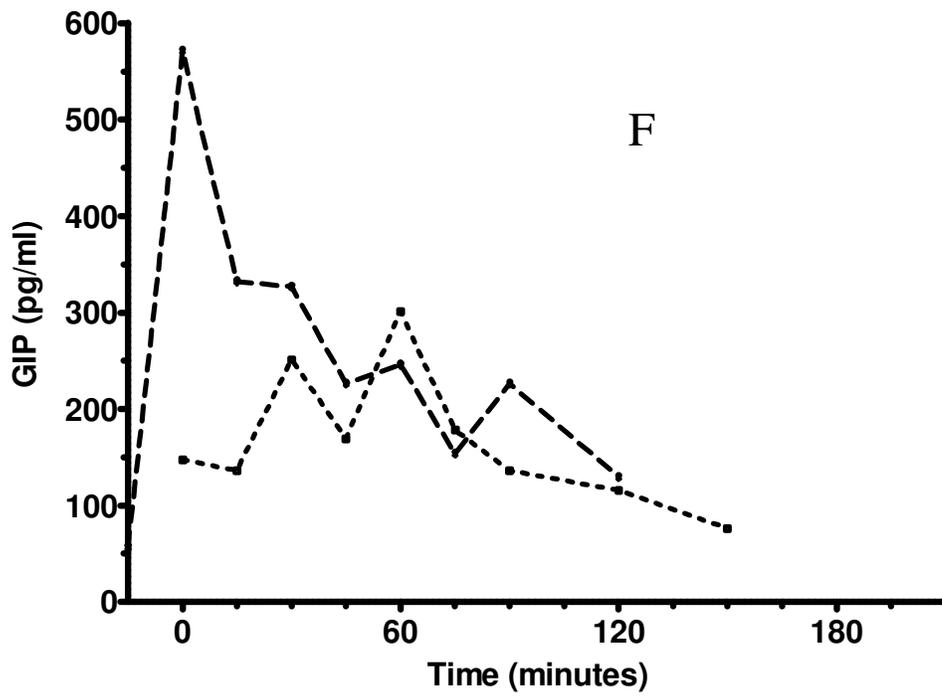
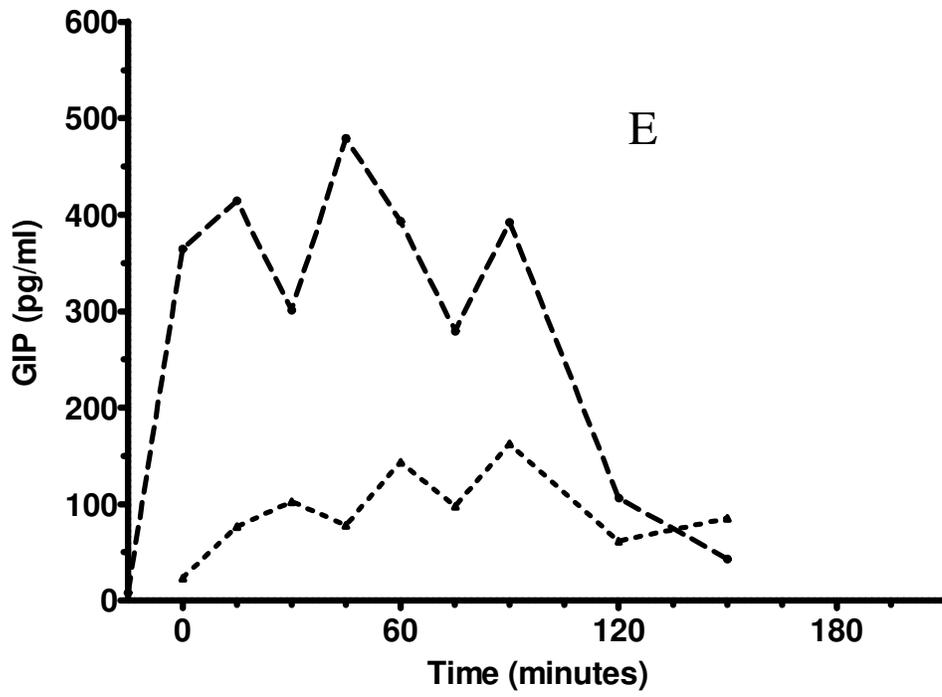


Figure 5.11 (continued)

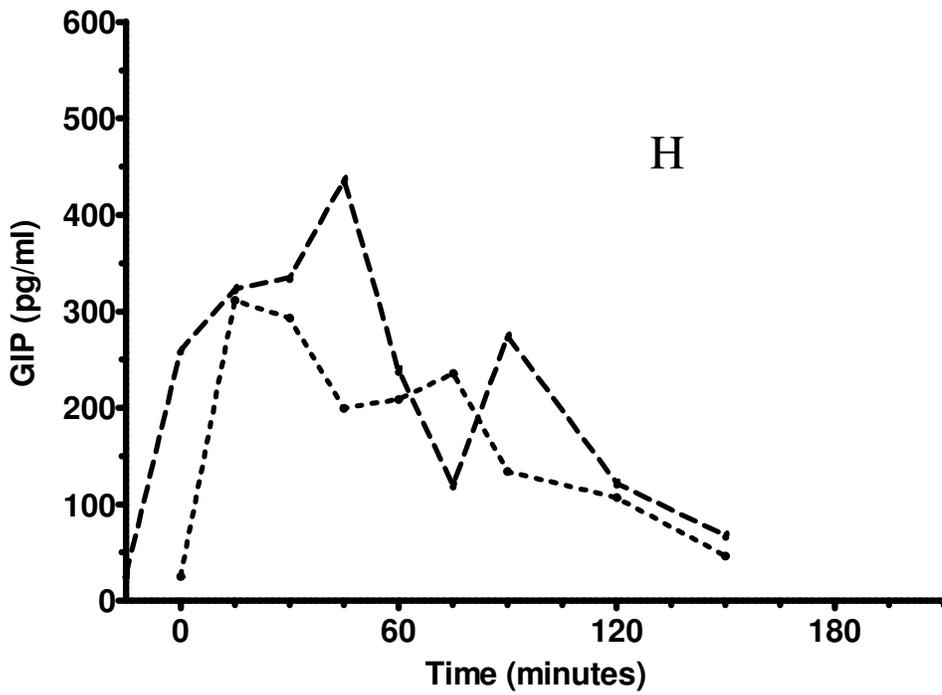
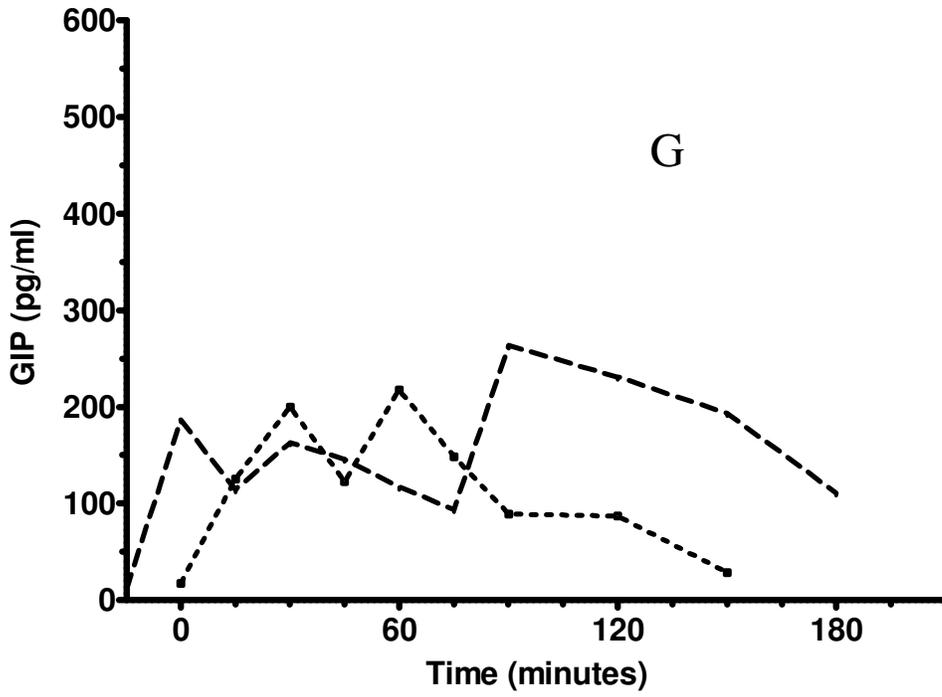
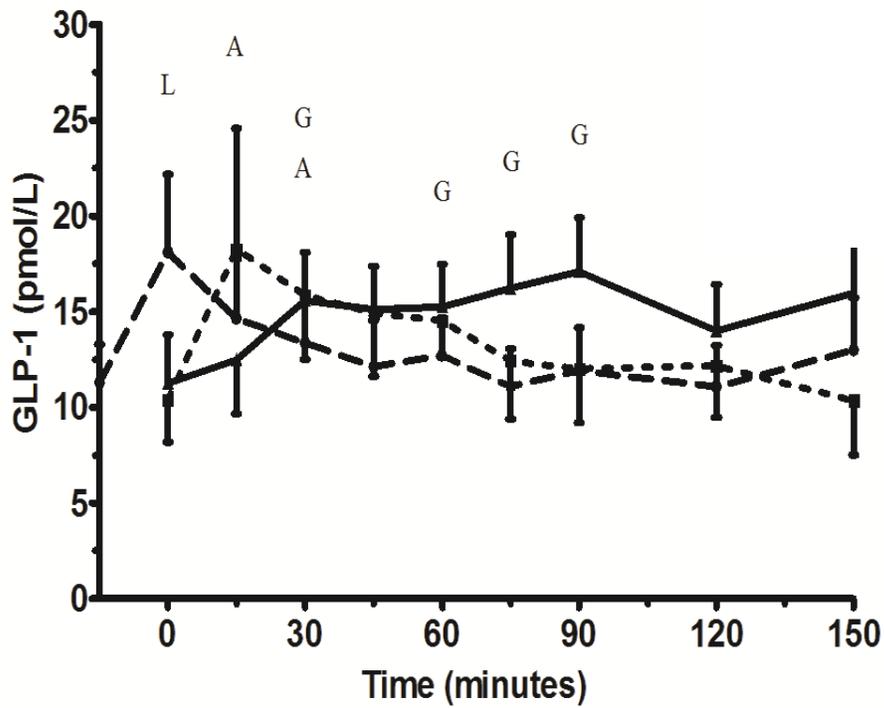


Figure 5.12 Serum total GLP-1 concentrations (pmol/L) during OGTT (solid line), LIGC (dashed line) and AAIGC (dotted line). Error bars represent the standard error of the mean. Letters indicate statistically significant difference from baseline of the respective experiment: G in OGTT, L in LIGC and A in AAIGC.



CHAPTER 6

THE GLP-1 MIMETIC EXENATIDE POTENTIATES INSULIN SECRETION IN HEALTHY CATS

Abstract

Background: The GLP-1 mimetic exenatide has a glucose-dependent insulinotropic effect and it is effective in controlling blood glucose with minimal side effects in people with type 2 diabetes. Exenatide also delays gastric emptying, increases satiety and improves beta-cell function.

Animals: Nine young, healthy, neutered, purpose-bred cats

Objectives: To study the effect of exenatide on insulin secretion in cats, during euglycemia and hyperglycemia.

Methods: Randomized, cross-over design. Blood glucose concentrations (BG) during an oral glucose tolerance test (OGTT) were determined in these cats previously. Two isoglycemic glucose clamps (mimicking the BG during the OGTT) were performed in each cat on separate days, one without prior treatment (IGC) and the second with exenatide (1 mcg/kg) injected subcutaneously 2 hours before (ExIGC). BG, insulin and exenatide were measured and glucose infusion rates were recorded and compared in paired-tests between the 2 experiments.

Results: Following exenatide injection, insulin serum concentrations increased significantly (2.4 fold [range 1.0 – 9.2, $P = 0.004$]) within 15 minutes. This was followed by a mild decrease in BG and a return of insulin concentration to baseline despite a

continuous increase in serum exenatide concentrations. Insulin AUC during ExIGC was significantly higher compared to insulin AUC during IGC (AUC ratio 2.0 ± 0.4 , $P=0.03$). Total glucose infused was not significantly different between IGC and ExIGC. Exenatide was detectable in plasma at 15 minutes post-injection. The mean exenatide concentration peaked at 45 minutes and then returned to baseline by 75 minutes. Exenatide was still detectable in the serum of 3 of 5 cats 8 hours post-injection. No adverse reactions to exenatide were observed.

Conclusion and clinical importance: Exenatide affects insulin secretion in cats in a glucose-dependent manner, similar to its effect in other species. Although this effect was not accompanied by a greater ability to dispose of an IV glucose infusion, other potentially beneficial effects of exenatide on pancreatic beta cells, mainly increasing their proliferation and survival, should be investigated in cats. Exenatide may not be useful as a glucose-lowering drug in diabetic cats.

Introduction

Type 2 diabetes mellitus is common in cats. Although diet change and oral medications may help initially, most diabetic cats depend on insulin therapy to survive (Nelson, 2005). Insulin therapy does not halt the progression of the disease and it has potential side effects. Weight gain may indicate good response to therapy initially but can eventually become a problem. Hypoglycemia is a common complication of insulin therapy and can be life-threatening (Nelson, 2005).

Glucagon-like polypeptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones. They are secreted from the gastrointestinal

tract into the circulation in response to ingestion of nutrients, and enhance glucose-stimulated insulin secretion. These hormones are responsible for the incretin effect in which oral glucose administration is associated with a much greater increase in plasma insulin concentrations when compared to the same amount of glucose given intravenously (McIntyre et al., 1965; Holst et al., 2009). In diabetics, the secretion of GIP is normal or slightly reduced but its insulinotropic effect on the pancreas is markedly impaired. In contrast, GLP-1 retains its insulinotropic effects in type 2 DM (at least at supraphysiologic concentrations), but secretion of GLP-1 is decreased (Nauck et al., 1993; Toft-Nielsen et al., 2001a, Toft-Nielsen et al., 2001b; Holst et al., 2009). The effects of GLP-1 and GIP extend well beyond potentiating insulin secretion. GLP-1 decreases glucagon secretion, increases satiety and slows gastric emptying. GIP increases glucagon secretion and enhances the action of insulin in adipose tissue. GLP-1 and GIP also increase proliferation of pancreatic beta-cells and decrease their apoptosis (Baggio and Drucker, 2007). Because of these beneficial effects, incretin-based drugs have been recently developed and successfully used as adjunctive treatments in human diabetic patients.

The peptide exendin-4 is a 39-amino acid peptide that shares 53% homology with GLP-1. It was first isolated from the poisonous venom of the Gila Monster (*Heloderma suspectum*) (Baggio and Drucker, 2007). Exendin-4 is a potent GLP-1 receptor-agonist but unlike GLP-1 it is not a substrate for dipeptidyl peptidase 4 (DPP-4) and neutral endopeptidase (NEP). DPP4 and NEP are ubiquitous in tissues and in plasma of people and rodents (Deacon, 2005). They are responsible for the fast degradation and short half-life of GLP-1 (a few minutes). Exenatide is a synthetic exendin-4. Resistant to

degradation, exenatide is eliminated by the kidneys and has a half-life of 3-4 hours in people. Its biological effect lasts about 8 hours after subcutaneous injection and it can be detected in the plasma for up to 15 hours (Kolterman et al., 2005). Exenatide has minimal side effects in people. It is sometimes associated with nausea and less frequently with vomiting. Infrequently, and especially when combined with other hypoglycemic drugs, it may cause hypoglycemia (Amori et al., 2007). Exenatide has been shown to be as effective as insulin glargine in the treatment of human type 2 DM but with less side effects such as weight gain and hypoglycemia (Heine et al., 2005; Barnett et al., 2007; Glass et al., 2008). In a 2-year follow-up of human patients receiving exenatide, patients achieved sustained and significant reductions in glycosylated hemoglobin, accompanied by significant weight loss and improvement in serum liver enzyme activity and blood pressure. Most importantly, treatment with exenatide improved beta cell function (as measured by HOMA-B) (Buse et al., 2007). Exenatide has also been used recently to improve the survival and function of transplanted pancreatic islets in type 1 DM (Ghofaili et al., 2007). These effects of exenatide on the function and survival of pancreatic beta cells open the door for halting the progression of diabetes as opposed to merely managing it.

Despite the crucial role incretins play in the pathogenesis and treatment of type 2 DM in people, and despite the similarities between DM in people and in cats and the high prevalence of this disease in both species (Rand et al., 2004), very little is known regarding incretins in cats. Immunoreactivity of GLP-1 and GIP has been described in enteroendocrine cells in cats but data regarding the stimulus for secretion of these hormones or their effects are scarce (Larsson and Moody, 1980; Vaillant and Lund,

1986). Recently it has been shown that GLP-1 concentrations increase in cats after intragastric administration of glucose (Hoenig et al., 2010). In another study, a DPP-4 inhibitor led to decrease in glucagon concentration and, to a lesser degree, to an increase in insulin concentrations after glucose challenge (Furrer et al., 2010). To the best of our knowledge, studies of incretin mimetics, such as exenatide, have not been reported in cats.

Incretins have been described in people, rodents, dogs, and other species. Although the sequence of GLP-1 is highly conserved, there are some differences among species in the distribution of the intestinal L cells that secrete GLP-1 and the degree of their stimulation by different nutrients and hormones (Damholt et al., 1999, Deacon, 2005). We hypothesized that in cats exenatide can potentiate insulin secretion without causing hypoglycemia. We also hypothesized that exenatide can be detected in plasma of cats for at least 8 hours after subcutaneous injection.

Materials and Methods

Animals: Nine young, healthy, purpose-bred cats were used in this study. There were 4 spayed females and 5 neutered males with a median age 56 months (range 38 – 58 months) and a mean body weight 4.8 ± 0.7 kg (range 3.8 – 6.1 kg). All cats were in normal body condition except one female that was slightly overweight. None of the cats were obese. Cats were group-housed in AAALAC-accredited facilities and all animal use was approved by the University of Illinois Institutional Animal Care and Use Committee. All cats were acclimatized and socialized for at least 4 weeks prior to the start of experiments, and extensive environmental enrichment was provided. Cats were

fed commercial cat food^a and monitored daily by physical exams. Body weight was measured weekly. Routine laboratory tests (including CBC, serum chemistry, serum total thyroxine, coagulation profile, and urinalysis) were performed at the time the cats were acquired and just prior to the first part of the experiment in each cat. PCV, plasma total solids and CBC's were repeated prior to the second part of the experiment and a CBC and a serum chemistry profile were repeated 2 weeks after exenatide injection in each cat.

Study design: A repeated-measures study design was used. An intravenous glucose infusion administered at a variable rate was used to mimic hyperglycemia as recorded during an oral glucose tolerance test (described in chapter 5). For each cat, blood glucose concentrations were measured every five minutes and the rate of infusion was adjusted to match the blood glucose that was recorded for the specific cats in a previous experiment. This isoglycemic glucose clamp was performed twice: On the first day with no other treatment (IGC) and 2 weeks later, 2 hours after an injection of exenatide^b (ExIGC). For glucose intravenous infusion, a 50% dextrose solution^c was diluted with saline to a 20% solution. Infusion rate was set on a syringe pump. Cats were fasted for 17 hours prior to each experiment (4:00 p.m. the afternoon before). Between 8:00 and 9:00 a.m. two blood samples for BG measurement were obtained. On one occasion in which hyperglycemia was detected, the experiment was postponed until the next day. In the IGC, glucose infusion started at 9:00 a.m. During the IGC, BG was measured at time zero and then every 5 minutes until euglycemia was restored. Blood samples for insulin, were collected at 0, 15, 30, 45, 60, 75, 90, 120, 150 and 180 minutes.

ExIGC: Performed 2 weeks after the IGC. At 9 a.m. (time zero of exenatide injection, -120 minutes from initiation of dextrose infusion) exenatide was injected using a 31g hypodermic needle that was attached to the pre-filled injection pen, as directed by the manufacturer. The pen delivers a fixed dose of 5 mcg (mean dose 1.04 ± 0.18 mcg/kg). The injection was given subcutaneously, in a previously shaved area in the mid abdomen. Two hours after the exenatide injection (zero minutes of the IGC), an IV dextrose infusion was started and an isoglycemic clamp was performed with BG target concentrations of the previous IGC. Blood samples for measurements of insulin and exendin-4 concentrations were obtained at time zero (exenatide injection), 15, 30, 45, 60, 75, 90, 120 (initiation of IGC), 135, 150, 165, 180, 195, 210, 240, 270 and 300 minutes. Samples were further obtained for exendin-4 measurements at 6, 8, 12 and 24 hours post injection. In one cat, the dextrose infusion was started at 60 minutes after injection because of hypoglycemia. In this cat, for statistical analysis, time 60 was considered time zero of the glucose infusion.

Blood collection and storage: Blood was collected through indwelling jugular catheters^d. The samples were collected into chilled glass tubes and then immediately centrifuged (at 4°C and 4000 RPM) and separated. Serum was stored at -20°C until analysis.

Catheter placement and maintenance: On the morning before each part of the experiment, cats were sedated with dexmedetomidine (0.009 mg/kg), butorphanol (0.22 mg/kg), and atropine (0.022mg/kg) to facilitate catheter placement. Jugular catheters were placed prior to the oral glucose tolerance test. These catheters were maintained by flushing heparinized saline daily until the end of the study. Cephalic catheters^e were

placed prior to IGC and ExIGC and were removed at the end of the dextrose infusion. These catheters were used exclusively for glucose infusion. A 1 sq inch area on the mid abdomen was shaved prior to the ExIGC to facilitate accuracy of subcutaneous injection and allow monitoring of the injection site. The sedation was reversed with atipemazole (0.009 mg/kg) and the cats were monitored until full recovery.

Glucose and hormone measurements: Blood glucose concentrations were measured with a hand-held point-of-care glucose meter^f that was validated for use in cats (Gilor et al., 2010). Insulin concentrations were measured with a feline ELISA^g. Exendin-4 was measured with a specific EIA kit^h. This assay has a range of 0 – 25 ng/ml and a sensitivity of 0.01 ng/ml. It has 100% cross reactivity with active exendin-4 (3 – 39) and its antagonist exendin (9 – 39) and 0% cross reactivity with GLP-1, GLP-2 and glucagon. The manufacturer of the assay reports an intra-assay CV of 5% and an inter-assay CV of 14% but in our laboratory the intra-assay CV was 12% and the inter-assay CV was 25%. Because this assay was not designed for use in cats we used pooled feline serum to replace the assay diluent in preparation of the standard curve so that potential matrix interference would be similar in the standard curve and in samples.

Statistical analysis: Statistical analysis was performed using computer softwareⁱ. The Shapiro-Wilk test was used to assess deviance from normal distribution of data. Grubbs' test was used to detect outliers. Data are reported as mean \pm SE if normally distributed or as median (range). Comparisons between treatment groups were analyzed with paired t-tests. Comparisons between time points were analyzed with one-way repeated measures ANOVA. Fold increase or fold difference were calculated as the

deviation of a ratio from 1.0 using a one sample t-test. The correlation between sets of abnormally distributed data was tested with Spearman's rank correlation coefficient rho.

The area under the curve (AUC) was calculated using the trapezoidal method for the BG and for insulin concentrations. AUC was used to represent total insulin secretion.

All statistical tests were performed as two-tailed tests. A P value < 0.05 was considered significant for all tests except the Grubbs' outlier test in which a P value < 0.01 was considered significant.

Results

Results of the OGTT and the incretin effect are reported in chapter 5.

In all cats, the injection of exenatide was not associated with any side effects and no local or systemic adverse reactions were observed. CBC's and serum chemistry profiles performed 2 weeks after exenatide injection showed no significant abnormalities in any of the cats.

Baseline BG did not differ significantly between the IGC and ExIGC (83.0 ± 2.9 mg/dl vs. 82.3 ± 3.3 mg/dl respectively, $P = 0.78$). Baseline insulin concentrations also did not differ significantly between the IGC and ExIGC (313 ± 42 ng/L vs. 301 ± 47 ng/L respectively, $P = 0.84$). Fifteen minutes after exenatide injection, insulin concentrations peaked at 724 ± 110 ng/L (an increase of 2.4 fold [1.0 – 9.2], $P = 0.004$, Figure 6.1). Insulin then returned to baseline at 30 minutes and decreased below baseline at 75 minutes (173 ± 48 ng/L, $P = 0.008$). This was associated with a trend towards a decrease in BG below baseline at 45 minutes (-6.9 ± 3.2 mg/dL, $P = 0.06$, Figure 6.2).

At 120 minutes (initiation of IV dextrose infusion), insulin concentrations and BG were not significantly different between the IGC and ExIGC ($P = 0.25$, $P = 0.4$ respectively). Blood glucose concentrations during IV dextrose infusion did not differ significantly between the IGC and ExIGC (Mean AUC difference $0.02 \pm 4\%$, $P = 0.95$, Figure 6.2). Total glucose infused also did not differ significantly between the IGC and ExIGC (0.49 ± 0.06 g/kg, 0.56 ± 0.09 g/kg respectively, $P = 0.35$, Figure 6.3). In only 6 cats, more glucose was infused during the ExIGC. In one cat, the amount of glucose infused during the ExIGC was almost double than the amount infused during the IGC. In this cat, BG decreased to 54 mg/dl one hour after exenatide injection and the glucose infusion was started at that time. Insulin AUC during IV dextrose infusion was significantly higher with exenatide injection compared to no treatment (AUC ratio 2.0 ± 0.4 , $P=0.03$, Figure 6.1). There was no correlation between the degree of stimulation at 15 minutes (calculated as the ratio of insulin concentration at 15 vs. zero minutes) and the overall effect (represented by the ratio of AUC of insulin concentration during glucose infusion in the ExIGC and IGC).

Exenatide pharmacokinetics: Exenatide was measured in 6 cats. One cat was excluded from analysis. In this cat, serum concentrations of exenatide were consistently very high and were calculated as outliers for all but 2 time points. Importantly, pre-injection concentrations were also very high, which suggested a matrix interference effect of this cat's serum. The results for the 5 other cats are presented in figure 6.4. The mean exenatide concentration increased at the first measurement (15 minutes post-injection) and reached a peak at 45 minutes post-injection ($P = 0.016$). By 90 minutes, the mean concentration was no longer significantly different from baseline. The mean

exenatide concentration from 120 to 240 minutes (during the isoglycemic clamp) was 0.4 ± 0.14 ng/ml. Exenatide was still detectable in the serum at 8 hours post injection in 3 out of 5 cats. The 15 minute exenatide concentration tended to have positive correlation with the fold increase of insulin concentrations from 0 to 15 minutes (Spearman's $\rho = 0.9$, $P = 0.083$).

Discussion

In this study we demonstrated that exenatide stimulates insulin secretion in a glucose-dependent manner in cats. A subcutaneous injection of exenatide caused a marked increase in serum insulin concentration within 15 minutes, followed by a small decrease in blood glucose concentrations and a return of insulin to baseline concentrations. The return of insulin to baseline concentrations occurred despite a continuous increase in exenatide concentrations in the serum. When exenatide concentrations peaked 45 minutes post-injection, BG was already below baseline and no further stimulation of insulin secretion occurred. When BG was elevated using a glucose infusion, the potentiating effect of exenatide was seen again. Serum insulin concentrations during the glucose infusion were significantly higher than serum insulin concentrations that were measured during an isoglycemic clamp.

The marked effect on insulin secretion at euglycemia was not unexpected. In isolated rat pancreas, GLP-1 and exenatide caused similar increases in insulin secretion at an ambient blood glucose concentration of 54 mg/dl (Parks et al., 2001). In healthy fasted people, a subcutaneous injection of GLP-1 caused a 5-fold increase in plasma insulin concentrations within 10 minutes, followed by a decrease in BG (Edwards et al.,

1998). Hypoglycemia developed in 3 of 10 subjects in that study with one of those becoming symptomatically hypoglycemic. Severe hypoglycemia is a rare side effect of exenatide in diabetic people and is always associated with concurrent treatment with sulfonylurea drugs (Amori et al., 2007). A single hypoglycemic event occurred in our study but glucose infusion was begun before clinical signs could have developed and its severity could not be assessed. Nausea, decreased appetite and less frequently, vomiting are observed in a subset of people with daily use of exenatide. No side effects were seen in our study despite giving approximately 10 times of the dose recommended in people.

In clinical use, subcutaneous exenatide injections demonstrate glucoregulatory and weight loss effects with sustained plasma concentrations in the 0.2-0.4 ng/ml range (Gedulin et al., 2008). In our study, during the glucose infusion the mean exenatide concentration in the 5 cats was 0.4 ng/ml. This was associated with a mild but significant effect on insulin secretion when compared to an isoglycemic clamp with no prior treatment. Despite the difference in insulin concentrations, the total amount of glucose that needed to be infused to maintain an isoglycemic clamp was not significantly different between the isoglycemic clamps. The mundane reason for that could be a lack of sufficient statistical power, however, a physiologic explanation should be considered. In general, if exenatide caused an increase in insulin concentrations with no net effect on blood glucose, it should have either increased hepatic glucose output or decreased glucose disposal. Neither of these potential effects has been described with GLP-1. Multiple studies, both *in vitro* and *in vivo*, have shown that exenatide in general has the same physiologic effects as GLP-1 (Kim and Egan, 2008). Recently, however, a study in rats revealed an unexpected consequence of acute exenatide administration (Perez-Tilve

et al., 2010). Exenatide, whether administered into the circulation or into the CNS, caused stimulation of the sympathetic nervous system, leading to hyperglycemia. This effect was independent of the glucose-dependent insulinotropic effect of exenatide and it waned with more chronic administration (after 6 days). A similar effect of exenatide in cats might cause an increase in hepatic glucose output and explain our results.

Importantly, it would also justify examining the effect of chronic exenatide administration in healthy and diabetic cats.

Increased hepatic glucose output might also be mediated through a direct effect on the pancreas to increase glucagon secretion. In general, GLP-1 inhibits glucagon secretion in a glucose dependent manner, thus reducing hepatic glucose output especially in hyperglycemia. In contrast, GIP directly stimulates glucagon secretion but this effect is usually overcome by the insulinotropic effects of GIP with a net suppression of glucagon secretion and an overall glucose lowering action (Kim and Egan, 2008). It is widely accepted that the effect of GLP-1 to inhibit glucagon secretion is mostly indirect, mediated by the insulinotropic effect of GLP-1. A direct effect of GLP-1 on alpha cells is questionable and in a recent study, GLP-1 receptors were not demonstrated in alpha cells of rodents and people (Tornehave et al., 2008). When GLP-1 receptor was expressed in alpha cells, it had an unexpected effect: stimulation of glucagon secretion (Dillon et al., 2005). Perhaps in cats, exenatide stimulates glucagon secretion (either via GLP-1 receptor or via a different mechanism), similar to the effect of GIP in alpha cells. If that is the case, the direct stimulatory effect of GLP-1 was not overcome by the insulinotropic effect. Our results suggest that exenatide in cats has a smaller insulinotropic effect compared to other species which might contribute to this overall

lack of glucose lowering effect. The hypothesis that exenatide stimulates glucagon secretion is not supported by the results of a recent study that showed in cats a reduction in glucagon secretion after injection of DPP-4 (Furrer et al., 2010).

Exenatide was detectable in plasma as early as 15 minutes post-injection (the earliest time point sampled), demonstrating its rapid absorption after a subcutaneous injection. The mean plasma exenatide concentration peaked 45 minutes post-injection and then sharply decreased, but exenatide was still detectable in the serum 8 hours post-injection in 3 out of 5 cats. These results are consistent with a prolonged half-life *in vivo* and a resistance to degradation by DPP-4 as is seen in other species (Kolterman et al., 2005). This is in sharp contrast to GLP-1, which is rapidly degraded after a subcutaneous injection with a half-life of 1–2 minutes (Baggio and Drucker, 2007). In this study we did not examine the biologic effect of exenatide throughout the duration of its presence in the serum, and, therefore, its duration of action could not be determined. Future studies should examine the duration of action of exenatide in cats as well as its effect on lowering blood glucose in diabetic cats. We have shown that exenatide has an insulinotropic effect in cats. Even if this effect is not translated into a glucose-lowering effect, as might be suggested from our isoglycemic clamp results, other biological effects of exenatide might be useful in the treatment of diabetes in cats and should be explored.

In conclusion, exenatide has a glucose-dependent insulinotropic effect in cats. Our study justifies the use of exenatide in a clinical trial in diabetic cats as well as further studies of novel long-acting GLP-1 analogs.

Footnotes

^a Purina DM, Nestlé Purina PetCare Company

^b Byetta® 5mcg injectable pen, Amylin Pharmaceuticals, Inc. San Diego, California, USA

^c 50% Dextrose USP. Hospira Inc. Lakeforest, IL USA

^d BD Angiocath AutoGuard®, Becton Dickinson Infusion Therapy Systems, Inc., Sand, UT USA

^e V-Cath®, 3.0F, NeoMedical, Inc, Fremont, CA USA

^f OneTouch Ultra, LifeScan Inc., Milpitas CA USA

^g Feline Insulin ELISA, Mercodia AB, Uppsala, Sweden

^h Exendin-4 EIA, Bachem Americas Inc., Torrance, California, USA

ⁱ GraphPad Prism, GraphPad Software Inc., CA, USA

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Figures

Figure 6.1 Serum insulin concentrations (ng/L) after exenatide injection (solid line) and during an isoglycemic clamp (broken line). Exenatide was injected at zero minutes. IV dextrose infusion was started at 120 minutes. Error bars represent the standard error of the mean.

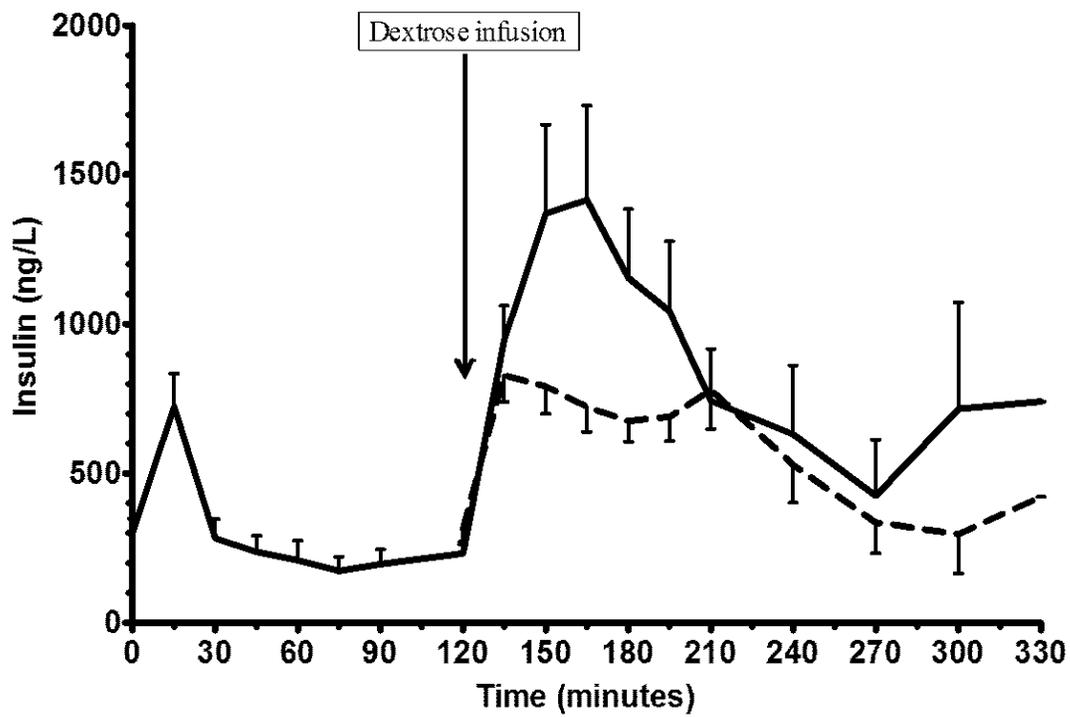


Figure 6.2 Blood glucose concentrations after exenatide injection (solid line) and during an isoglycemic clamp (broken line). Exenatide was injected at zero minutes. IV dextrose infusion was started at 120 minutes. Error bars represent the standard error of the mean.

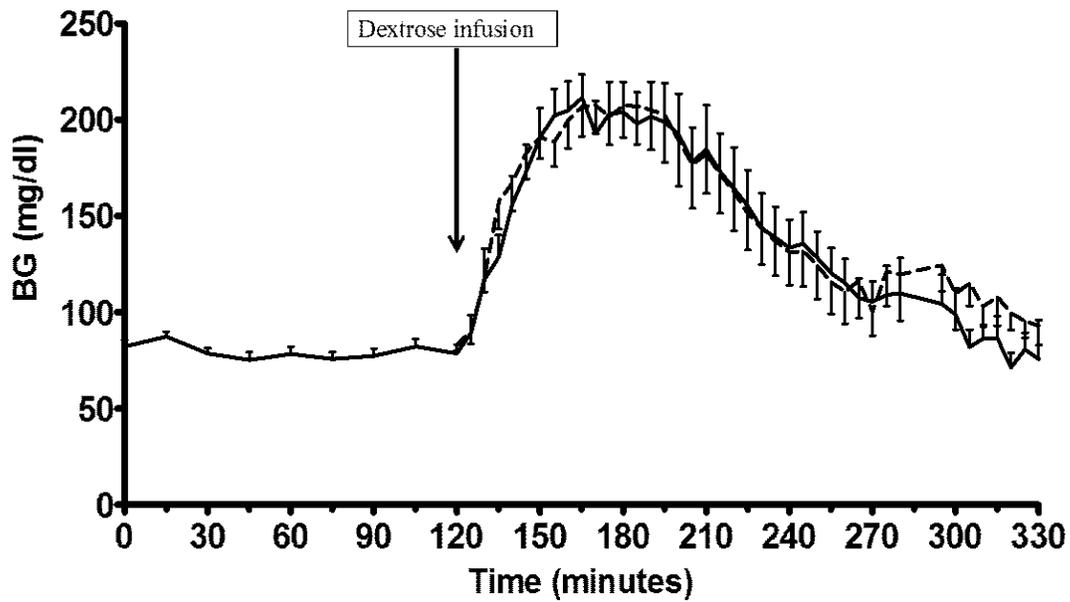


Figure 6.3 Total glucose infused during IGC and ExIGC in individual cats in paired experiments.

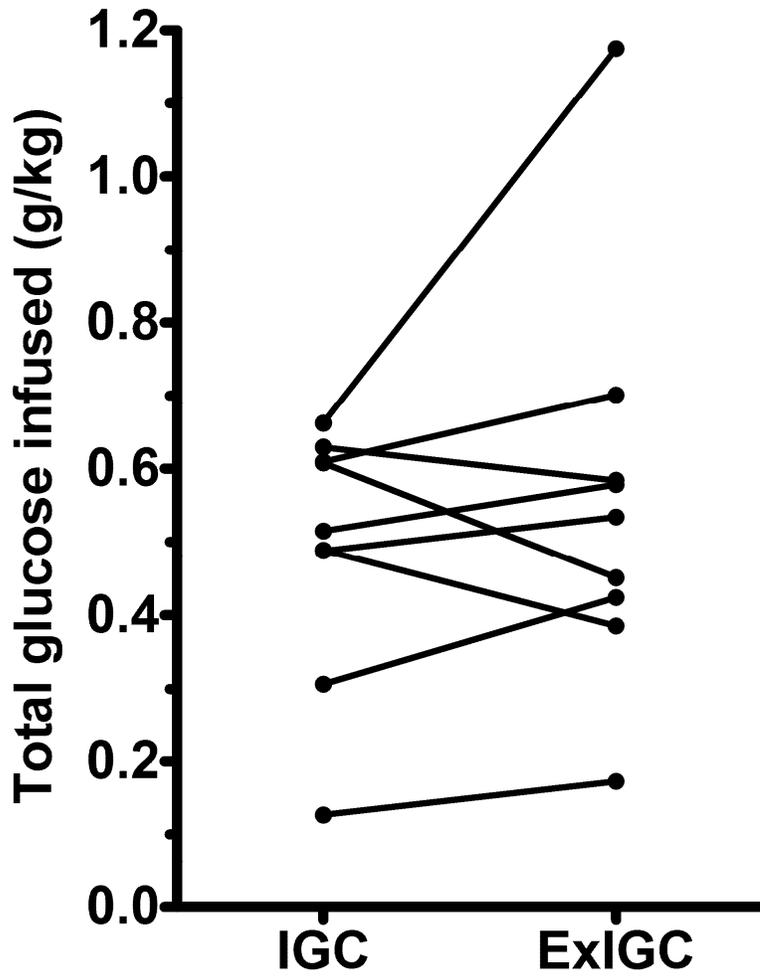
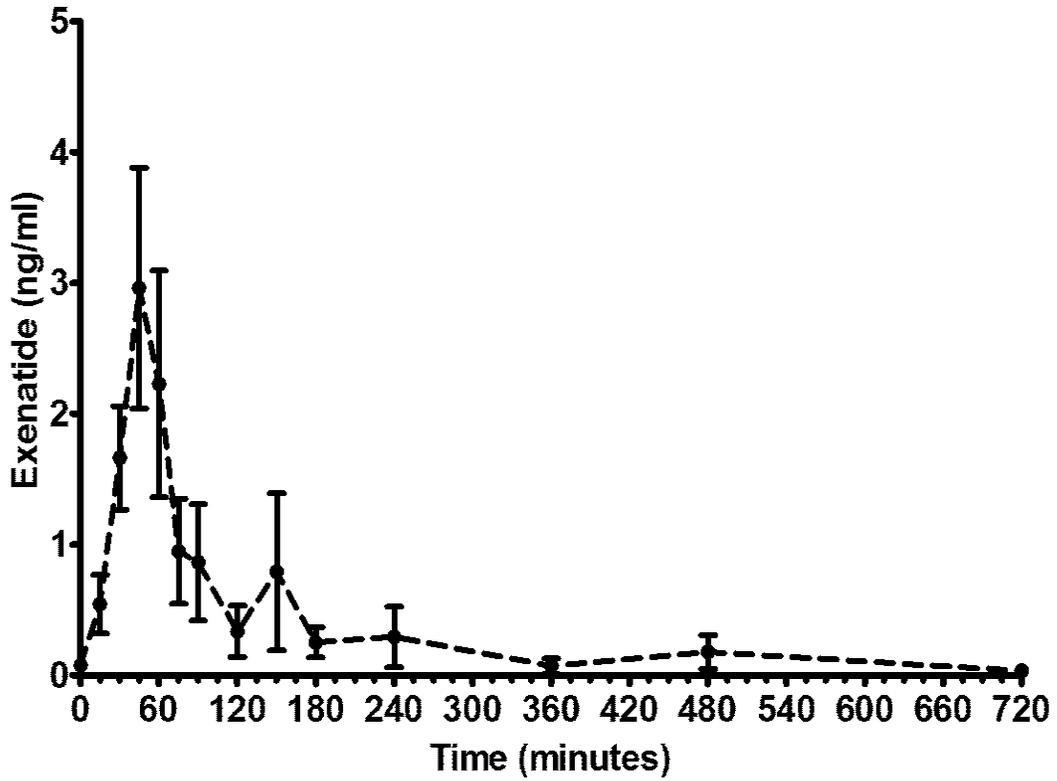


Figure 6.4 Exenatide concentrations (ng/ml) after a subcutaneous injection at zero minutes. IV dextrose infusion was started at 120 minutes.



CHAPTER 7

SUMMARY

Diabetes is a common disease in middle-aged and older cats. With a prevalence of 0.5 - 2% (Panciera et al., 1990; Rand et al., 2004) and an estimated population of 90 million cats in American households alone, there are approximately 1 million diabetic cats in the United States. Recent evidence suggests that the prevalence of diabetes in cats is increasing because of an increase in the frequency of predisposing factors such as obesity and physical inactivity (Prahl et al., 2007). Diabetes is also a common reason for euthanasia of feline patients. This is partly because of the low compliance of owners to twice-a-day injections of insulin, and partly because of the complications that are associated with the disease and its treatment. In people, novel treatments for diabetes have proven useful in improving beta cell function and glycemic control, and, at the same time, reducing the rate of side effects of treatment. We studied some of these treatments in healthy cats. The data presented here justify clinical trials of these drugs in diabetic cats. Our data also give insights into the unique physiology of the domestic cat and can serve as a basis for new avenues of research in treatment of diabetes and obesity by diet manipulation.

Type 2 diabetes mellitus is characterized by an insufficient secretion of insulin in response to hyperglycemia. Although diet change and oral medications may help initially, most diabetic cats depend on insulin therapy to survive (Nelson, 2005; Marshall

et al., 2008). Hypoglycemia is a common complication of insulin therapy and can be life-threatening (Nelson, 2005). The occurrence of hypoglycemia has been reduced significantly in human medicine with the introduction of the synthetic insulin analogs insulin detemir and insulin glargine (Danne et al., 2008; Fakhoury et al., 2008; Monami et al., 2008). These insulin analogs have long durations of action with a relatively flat time-action profiles, and, thus, are used as once-a-day drugs to replace basal secretion of insulin. We compared the pharmacodynamics of insulin detemir and insulin glargine in healthy cats using the isoglycemic clamp method. We also studied the pharmacokinetics of these insulin analogs in cats. We found that the duration of action of both analogs is shorter than is reported in people. With a mean duration of action of about 12 and 10 hours, respectively, insulin detemir and insulin glargine should probably be used as twice-a-day drugs in cats. It has been shown in people, however, that in patients with type 2 diabetes a basal insulin that does not last 24 hours can sometimes be sufficient because of residual endogenous insulin secretion (Swinnen et al., 2008). A small clinical trial in cats has shown that clinical signs of diabetes can be controlled with once-a-day insulin glargine (Weaver et al., 2006). Therefore, the efficacy of insulin detemir and insulin glargine should be compared in a clinical trial in diabetic cats. In people, prolongation of the duration of action of peakless insulin analogs can be achieved by increasing doses (Plank et al., 2005; Klein et al., 2007). We showed that the pharmacodynamic profiles of insulin detemir and insulin glargine are variable, and “peakless” time-action profiles cannot be safely assumed. Thus, increasing the dose to achieve longer duration of action might result in an unwanted higher peak of action and a hypoglycemic crisis.

Traditionally, duration of action and potency of an insulin formulation are assessed with blood glucose curves. These require frequent blood sampling and they rely on the unfounded assumption that day-to-day variability of the insulin formulation is minimal. Of commercially available insulin formulations, insulin detemir has the lowest day-to-day variability in people (Klein et al., 2007). Future studies should investigate this in cats and may prove the usefulness of glucose curves in monitoring diabetic patients. In our comparison of the pharmacodynamics and pharmacokinetics of insulin detemir, we found that a glucose-lowering effect can no longer be observed after the measured insulin concentration (unadjusted for insulin cross reactivity) decreased below 25 mU/L in the Iso-Insulin ELISA. This information might be clinically useful. If measured after an overnight fast, one single measurement of serum insulin concentration in the Iso-Insulin ELISA could indicate whether insulin detemir is still present in concentrations that should have a glucose-lowering effect, and could give an indication of its duration of action. This might replace the cumbersome and inaccurate blood glucose curve method, especially if insulin detemir proves to have a low day-to-day variability in diabetic cats.

Insulin therapy, despite the side effects associated with it, is necessary for survival in insulin-deficient patients. Most type 2 diabetic people and many type 2 diabetic cats retain some residual capacity to secrete insulin, albeit in insufficient amounts. Incretin-based therapies take advantage of this phenomenon. The incretin hormones GIP and GLP-1 stimulate insulin secretion in a glucose-dependent manner. Thus, they are less likely to cause hypoglycemia than exogenous insulin. Incretin hormones also stimulate insulin biosynthesis and beta cell proliferation and improve beta cell survival. Incretin-

based therapies are as effective as insulin therapy in treating diabetes in people but they are associated with fewer side effects.

We studied the synthetic GLP-1-mimetic exenatide in healthy cats. We used the only commercially available formulation of exenatide (at present) which is supplied as a pre-filled injection pen. The pen delivers a fixed dose of 5 mcg so that the final dose administered in this study was about 10 times higher than the recommended dose in people. We found that exenatide stimulates insulin secretion in cats in a glucose-dependent manner, but it did not increase glucose tolerability. It is plausible that, as in rats, exenatide also has stimulating effects in the sympathetic nervous system, thus increasing hepatic glucose production (Perez-Tilve et al., 2010). In rats the acute effect on the sympathetic nervous system wanes off after a few days but the insulinotropic effect remains. If this is also the case in cats, exenatide might still be effective in lowering blood glucose concentrations in diabetic patients. We also found that exenatide is rapidly absorbed after a subcutaneous injection and it is rapidly cleared from the blood. Finally, one out of nine cats in this study developed hypoglycemia. The lack of apparent glucose-lowering effect, the rapid clearance from the blood and the potential safety concern, make this formulation of exenatide of questionable advantage in the treatment of feline diabetes. Our data do show, however, that this incretin-mimetic has insulinotropic effects in cats, which means that, in principal, incretin-mimetics should be studied further in cats. A different formulation, one that is longer-acting and with more flexibility in dosing, might prove beneficial.

Finally, we studied some physiologic aspects of the incretin effect in cats. By definition, the incretin effect is the secretion of an excess amount of insulin after oral

ingestion of glucose when compared to intravenously administered glucose. Incretin hormones, however, are secreted in response to ingestion of other nutrients as well and they have a multitude of other effects. We asked two questions in this study: First, is there an incretin effect, as defined above, in cats?, and second, what is the differential effect of oral glucose, lipids, or amino acids on secretion of GIP, GLP-1 and insulin? To answer the first question, we performed an oral glucose tolerance test and compared the results to an isoglycemic clamp in each cat. To the best of our knowledge, this is the first report of an oral glucose tolerance testing in awake cats. Because stress has an effect on blood glucose and insulin secretion, nasoesophageal tubes, placed on the day before each experiment under light sedation, were used for oral administration of glucose. We also report here, for the first time, the fasting and stimulated serum concentration of GIP in cats.

We found that a glucose-stimulated incretin effect does occur in cats, it is probably mediated by GLP-1, and its magnitude is lower than what has been reported in other species (Nauck et al., 1986). This small incretin effect is probably related to the fact that GIP secretion was not stimulated by glucose. This is a unique finding that contrasts with reports from other species, but it is not surprising given the fact that cats are obligate carnivores and do not feed on carbohydrates in the wild. In concordance, GIP was strongly stimulated by amino acids and even more so by lipids. GLP-1 secretion was stimulated to a similar degree by all three nutrients. It is plausible that insufficient activity of GIP after a meal rich in carbohydrates might contribute to prolonged postprandial hyperglycemia and the development of diabetes. Interestingly, insulin secretion was stimulated in this study after fat ingestion when blood glucose

concentrations were normal. This was probably the result of stimulation by GLP-1 and/or GIP. We also demonstrated stimulation of insulin secretion at euglycemia by exenatide as described in chapter 6. The stimulation of insulin secretion by exenatide ceased when blood glucose decreased about 10% below baseline, despite a continuous rise in exenatide concentrations. This stimulation of insulin secretion at euglycemia might explain the observation that in cats fed a diet rich in protein and fat (and low in carbohydrates) post-prandial blood glucose decreases (Mori et al., 2009).

Our data support further studies of the use of insulin detemir and insulin glargine in diabetic cats, and further investigation of the plausibility of monitoring insulin detemir treatment by measuring insulin concentration in the blood. Our data do not support the clinical use of the GLP-1-mimetic exenatide in its currently available formulation, but our data do support further investigation of similar drugs with longer durations of action. Finally, we report new findings on the incretin effect in cats. GIP secretion is not stimulated by oral glucose in cats but it is strongly stimulated by amino acids and lipids. GLP-1 mediates a glucose-stimulated incretin effect in cats, and its secretion is similarly stimulated by glucose, amino acids, and lipids.

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AUTHOR'S BIOGRAPHY

Dr. Chen Gilor was born to Yehoshua and Ronit Gilor on May 5, 1972 in Haifa, Israel. He was raised in the town of Kiriath Bialik, Israel. After graduating from high school in 1990 he went on to attend the Faculty of Agriculture in the Hebrew University of Jerusalem, majoring in Animal Sciences. In 1993, after earning a place in the Dean's list of excellence for 2 years in a row, Chen was one of 20 lucky students to be accepted to the Koret School of Veterinary Medicine in the Hebrew University of Jerusalem, where he received his Doctorate of Veterinary Medicine degree in 1997. He then served as a veterinary officer in the Israeli Defense Force for 3 years while working in his free time as a general practitioner in a small animal clinic in Tel Aviv. Chen got married in 1997 to Shir who was then a student of veterinary medicine. He first visited in the USA in 1997 as an extern student in the internal medicine service at the University of Pennsylvania. On his second visit to the USA in 2001, together with his wife Shir, he spent a month as a preceptor at the Animal Medical Center in NYC, NY. Later that year they both visited the USA again, spending a month at the College of Veterinary Medicine in Cornell University. They continued from there to bag back in South America for 6 months until June of 2002 when they settled in Manhattan, NYC. Chen worked as a general practitioner for almost 2 years, first in a small animal emergency hospital in Staten Island and then as a general practitioner in a small animal clinic in Queens. He then spent a rigorous year as an intern at the Animal Medical Center, NYC, NY but not before attending a language school for a month in Maceio, Brazil, polishing his Portuguese and his affection to Brazilian culture. In July of 2005, Chen started a

Masters degree combined with a Small Animal Internal Medicine residency program at the University of Illinois in Urbana-Champaign. In 2007 Chen was awarded The Ann Johnson and Wally Hoffman Resident Teaching Excellence Award. In 2008 he won the Virbac Animal Health 4th Annual Award for best endocrinology abstract he presented at the ACVIM annual meeting in San Antonio, TX. At the same meeting he took his board certifying exam and became a Diplomate of the American College of Veterinary Internal Medicine. Also that year, while completing his residency, Chen won the Brasley Fellowship Award at the College of Veterinary Medicine, University of Illinois, and stayed at the U of I as a research fellow to work towards his Ph.D. Throughout his residency and his graduate studies, Chen was mentored by Dr. Thomas Graves. Chen's research was focused at first on the early diagnosis of diabetes in cats. This included laboratory work studying beta cell function in healthy cats as well as a clinical study on the correlation of fructosamine concentrations and obesity. His Ph.D dissertation revolved around the pharmacology of insulin analogs and the GLP-1 mimetic exenatide, as well as the physiology of incretin hormones in cats.

Chen and Shir's first son, Tom, was born in Urbana Champaign on February 2, 2009.