MECHANISMS OF BETA CELL DYSFUNCTION AND APPLICATION OF GLP-1 MIMETICS IN FELINE DIABETES MELLITUS

By

AARON MICHAEL HERNDON

Bachelor of Veterinary Science
Texas A&M University
College Station, Texas
1995

Doctor of Veterinary Medicine
Texas A&M University
College Station, Texas
1998

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Oklahoma State University
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Dissertation Approved:

Dianne McFarlane, DVM, PhD, DACVIM

Dissertation Adviser

Lara Maxwell, DVM, PhD, DACVP

Melanie Breshears, DVM, PhD, DACVP

Andrew Hanzlicek, DVM, MS, DACVIM

Arpita Basu, MS, PhD, RD

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Name: AARON MICHAEL HERNDON

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Abstract:

The causes of beta cell dysfunction and loss in feline diabetes mellitus (FDM) are incompletely understood. Potential causes of beta cell failure include amyloid accumulation, oxidative stress, inflammatory cytokine signaling, and endoplasmic reticulum (ER) stress. GLP-1 and GLP-1 mimetics have been proposed to have a beta cell sparing effect by mitigating cellular stress and pro-apoptotic signaling resulting from these causes, thereby inhibiting cell death. The purpose of the work described herein was three fold: to identify cellular mechanisms associated with beta cell dysfunction in FDM, to develop techniques for the use of freshly isolated feline islets for the study of islet cell dysfunction in cats, and to study the mechanisms and utility of GLP-1 mimetics as a therapy of FDM. Firstly, we tested the hypothesis that the pancreatic islets of cats with FDM have increased inflammation, oxidative stress, and pancreatic amyloid as compared to apparently healthy control cats. The results of this study suggest that oxidative modification and inflammatory cytokine signaling are present in the normal feline islet and increase with obesity and hyperglycemia. Secondly, we developed techniques for the isolation and culture of fresh feline pancreatic islets for in vitro study. Viability of islets was maintained for at least 5 days. An inconsistent response to glucose stimulated insulin production and release was seen in isolated islets, suggesting a lack of normal physiologic responsiveness of the islets. Thirdly, ER stress was induced in freshly isolated islets and the protective effect of the incretin mimetic exenatide was evaluated. Exenatide treatment resulted in a reduction of apoptotic signaling secondary to induced ER stress. Lastly, the clinical utility of incretin mimetics as an adjunct therapy for FDM was tested in a clinical trial evaluating insulin alone versus insulin with adjunct exenatide. Exenatide was not associated with improved glycemic control in cats, but results suggest a potential benefit of weight loss in obese diabetic cats. The study was limited by the small sample size resulting in a statistically underpowered study. Investigations in a larger population of diabetic cats are needed to assess the potential benefits of exenatide in the treatment of FDM.

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INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The incidence of feline diabetes mellitus (FDM) is as much as 15 times higher now than what was reported 40 years ago, making it one of the most common endocrinopathies seen in domestic cats. The disease has significant potentially negative effects on patient quality of life, the human-animal bond shared with the patient's owner, and overall patient longevity. The causes of this increase in disease incidence are incompletely understood.

Diabetes mellitus is the clinical manifestation of a failure of glucose homeostasis resulting in uncontrolled hyperglycemia. Although the mechanisms underlying this failure are varied and involve the interplay between many different neurohormonal pathways, it is ultimately the failure of insulin activity on sensitive tissues that results in the major clinical manifestations.

The cellular mechanisms leading to a lack of insulin effectiveness involves decrease of peripheral insulin sensitivity in tissues such as adipose or skeletal muscle and the inability of pancreatic beta cells to produce sufficient hormones to elicit a peripheral effect. Insulin is the primary secretory product of pancreatic beta cells found within the islets of Langerhans. Beta cells sense changes in glucose in the interstitial space and insulin release is promoted in the face of rising glucose and inhibited in times of decreasing glucose.

1.2 Epidemiology of feline diabetes mellitus

Several studies have investigated the incidence of FDM. It was estimated that the incidence of feline diabetes was 0.08% in 1970, but by recent accounts is now estimated to be as high as 1.2%.[1,2]

Feline diabetes mellitus and type 2 diabetes mellitus in people share many striking similarities. Both diseases are rooted in an inherent resistance of peripheral tissues to the effects of insulin and a subsequent period of hyperinsulinemia. Both are associated with so-called "life style" factors such as poor diet, inactivity, and obesity. In both diseases, it is pancreatic beta cell exhaustion that ultimately leads to uncontrolled hyperglycemia and clinical signs. In many cases, early detection and reversal of these lifestyle factors may result in remission of disease and return to euglycemic control.

There are many possible explanations for the increase in diagnosed cases of feline diabetes. Insulin resistance leading to an increased burden on the pancreas to maintain euglycemia appears to be central to the development of both type 2 diabetes in people and feline diabetes. The effects of obesity on the prevalence of diabetes cannot be overstated and an epidemic of obesity in both people and cats is well documented.[3,4] Obesity in cats is strongly associated with feline diabetes, with obese cats being 3.9 times more likely than lean cats to develop diabetes.[5] It is well documented that obesity in people is associated with decreased insulin sensitivity. The same correlation has been made in multiple experiments in cats. In one study cat study, weight gain of as little as 1kg was associated with a 30% decrease in insulin sensitivity.[6] In another study, an increase in body weight of 44% resulted in a 52% decrease in insulin sensitivity.[7]

It is also evident that genetic predispositions are associated with development of feline diabetes. The increased incidence of diabetes in Burmese cats (2.9-3.7 times more likely compared to other breeds) suggests a genetic predisposition in this breed.[8,9] Furthermore, a

polymorphism in the melanocortin 4 receptor gene has been positively correlated with frequency of diabetes in both obese and lean cats.[10]

1.3 The anatomy of the Islet of Langerhans

Named for the German medical student who identified islands of cells within the exocrine pancreas that did not appear to be responsible for the digestion of food, Islets of Langerhans comprise approximately 3% of the cat pancreas by weight.[11] Islets, regardless of species or location within the pancreas, are comprised of 5 primary endocrine cell lines, each with it's own secretory hormone products: alpha (glucagon), beta (insulin, islet amyloid polypeptide), delta (somatostatin), epsilon (ghrelin), and PP (pancreatic polypeptide). Islets are not homogenous in their size and cellular makeup. They can range from as few as a dozen cells to several thousand cells.[12] In the cat, the cells comprising the largest percentage of the islet are the beta cells, which comprise roughly 60% of the volume of the islet and are found typically along the periphery with some clusters within the interior of the islet.[12] (Figure 1.1) This is in contrast to mouse islets, which are composed of nearly 80% beta cells that are arranged within the core of the islet. Human islets are roughly 50% beta cells and arranged more randomly than is reported for both cats and mice.[12]

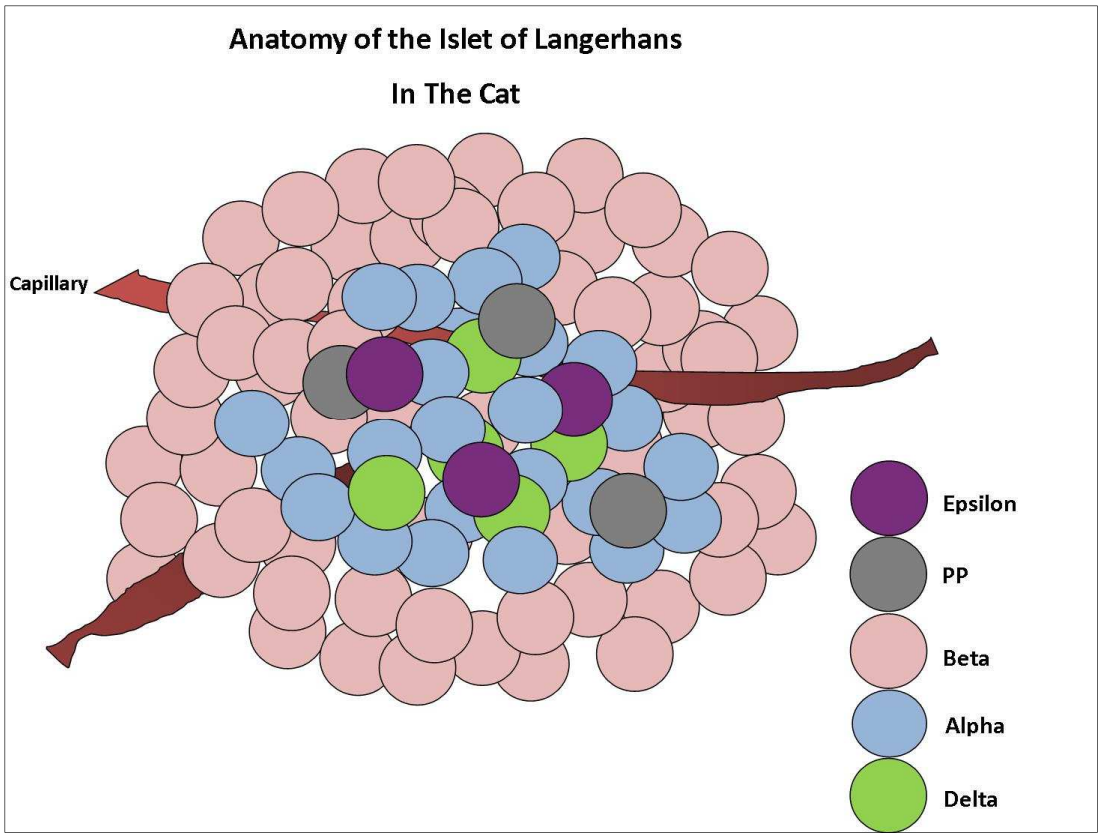

Figure 1.1. The feline islet is comprised of 5 primary cell types. The beta cells tend to form a ring, or mantle, around a core of alpha, delta, epsilon, and PP cells. Islets have rich capillary blood flow.

1.4 Neuroendocrine regulation of euglycemia

Neuroendocrine control of serum glucose is largely dependent on insulin activity, but also relies heavily on the effects of glucagon, islet-associated polypeptide, incretins (e.g. gastric inhibitory peptide and glucagon-like peptide-1), and the sympathetic nervous system.

1.4.1 Secretory products of the beta cell: insulin

Insulin is the primary secretory product of the pancreatic beta cells. Regulation of expression of the insulin gene is complex, and under the control of many different binding elements in the insulin promoter region. Glucose-regulated promotion of insulin gene transcription is largely under the influence of pancreatic and duodenal homeobox-1 (PDX-1), neurogenic differentiation 1(NeuroD1), V-maf musculoaponeurotic fibrosarcoma oncogene homologue A (MafA), and Pax6.[13-15] Also, products of the MAPK system and CRE binding elements are all potent promoters of insulin expression.[13] Inhibitors of insulin expression include c-Jun and CCAAT-enhancer binding protein beta (c/EPBβ).[14] The specific mechanisms associated with promotion or inhibition of insulin production by individual hormones or drugs are discussed in those respective sections. (Figure 1.2)

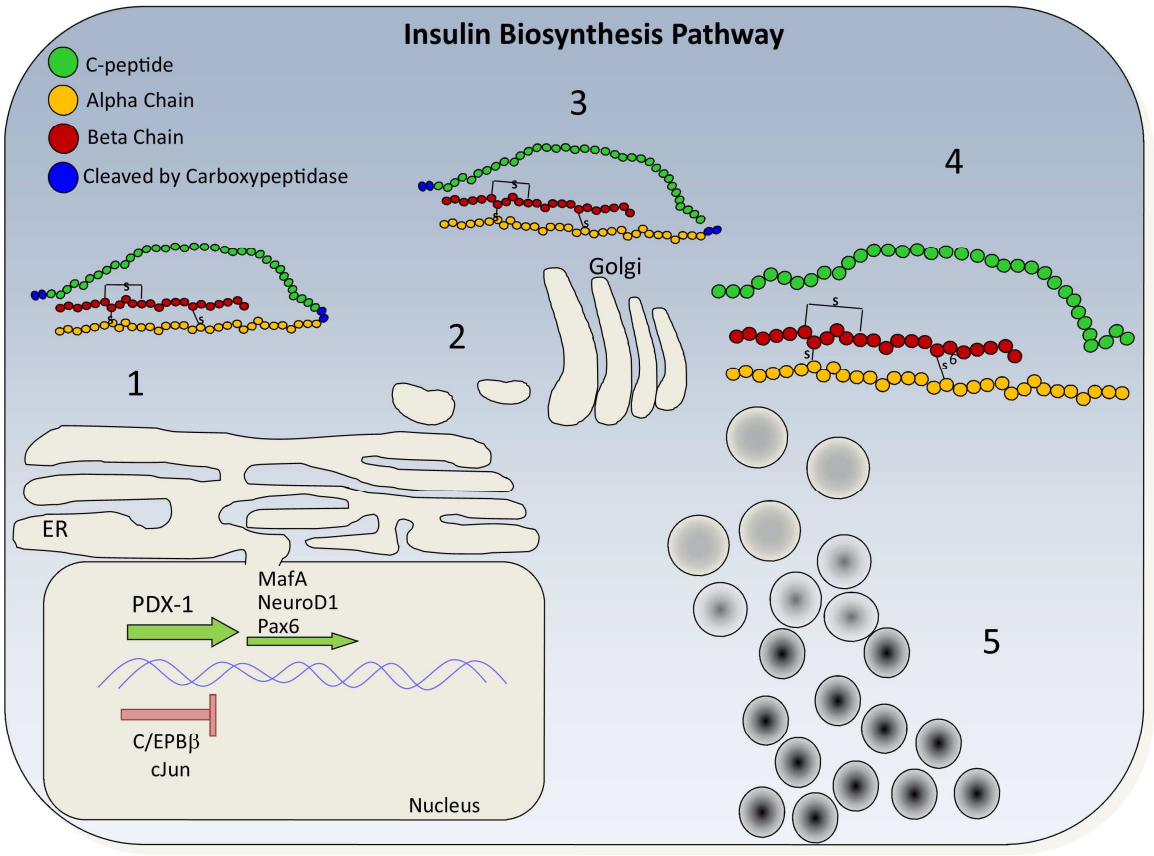

Figure 1.2. Biosynthetic pathway of insulin in the beta cell. 1: PC1 begins the process of separating the alpha and beta chains fro the c-peptide during transport through the ER. 2: Proinsulin is chaperoned from the ER to the golgi. 3: During transit through the golgi network, PC2 activity further separates the c-peptide from the alpha and beta chains while three disulfide bonds are formed. 4: Newly formed secretory vesicles are lower in density. The continued activity of PC2 and carboxypeptidase-E complete the processing of insulin, resulting in mature insulin consisting of an alpha and beta chain (joined by two disulfide bonds) as well as the c-peptide chain. 5: as vesicles mature, they increase in density as insulin crystalizes to form the more dense core of the organelle.

Initial processing is completed by prohormone convertase 1/3 (PC1) within the endoplasmic reticulum. As the protein is chaperoned through the *trans*-golgi network, prohormone convertase 2 (PC2) provides the second enzymatic cleavage of proinsulin. Once packaged inside the secretory vesicle, the actions of carboxypeptidase-E (in concert with PC2) provide the final modification, releasing the c-peptide fragment from insulin, which is now a 5.8kD protein comprised of a 21 amino acid alpha chain and a 30 amino acid beta chain with three disulfide bonds.[16] Both of these products (insulin and c-peptide) are released in equimolar concentrations. (Figure 1.2)

Secretory vesicles increase in density as their core becomes more acidified and the insulin monomers precipitate into a crystalline pentameric structure. Within the vesicles, insulin crystals are stabilized by interactions with zinc (and to a lesser extent islet associated polypeptide) at a low pH (pH 5.5).[17] Insulin crystals make up the majority of the dense core of the granule, with c-peptide and IAPP molecules comprising much of the halo region around the core.[18]

Docking of secretory granules and exocytosis is SNARE-dependent and initiated by increases in cellular calcium and membrane depolarization. The primary stimulation for insulin release is an increase in the ATP:ADP ratio in the cell. ATP concentration increases in response to an influx of glucose into the beta cell (via the bi-directional and constitutively expressed GLUT-2 receptor). Higher concentrations of ATP trigger inactivation of the potassium/ATP pump, leading to membrane depolarization and opening of the voltage-gated calcium channel in the cell membrane. The membrane-bound potassium/ATP channel is also inactivated by increased cytosolic cyclic AMP which is the result of adenylyl cyclase activation secondary to signaling from adrenergic, GIP, and GLP-1 receptors.[16] (Figure 1.3)

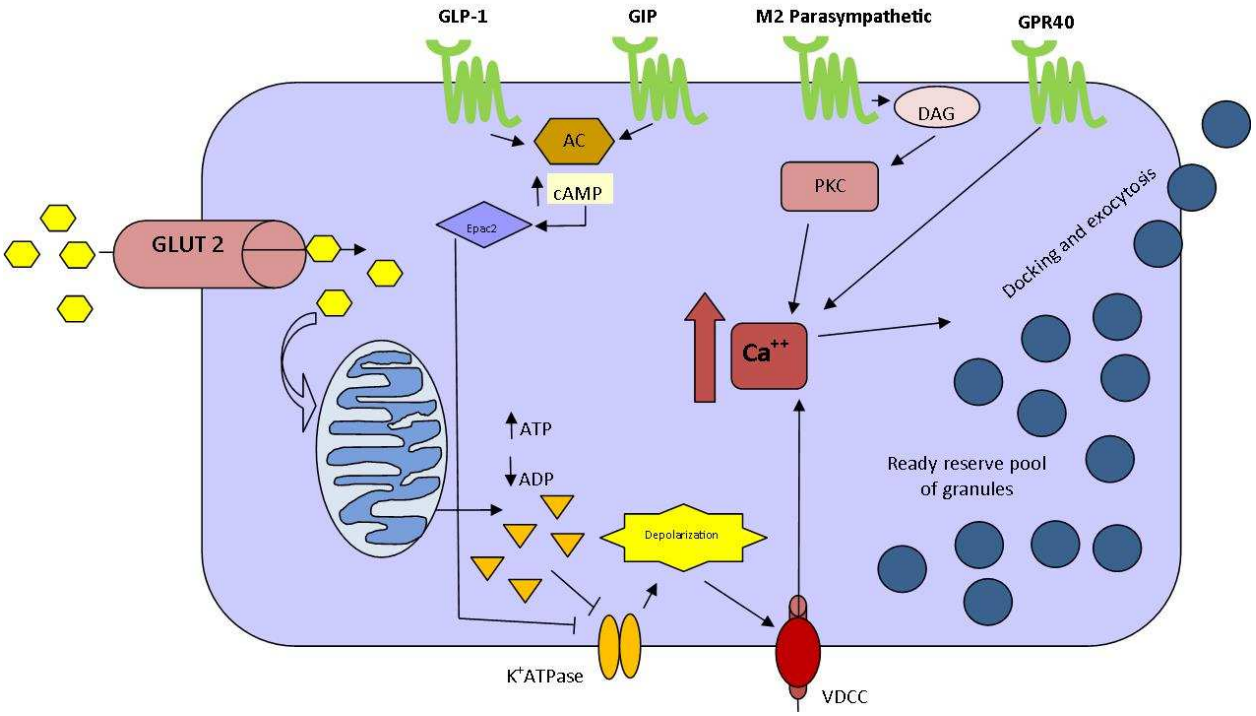

Figure 1.3: Increases in cytosolic calcium trigger docking and exocytosis of beta cell granules. Beta cells "sense" glucose in the interstitial space due to the high-affinity, bi-directional nature of the GLUT-2 transporter. Increases in ATP/ADP ratio secondary to mitochondrial respiration induce a closure of the potassium ATPase transport which initializes depolarization and opening of the voltage-dependent calcium channel. Both GLP-1 and GIP activate adenylyl cyclase and the increase in cytosolic cAMP activates the Epac2 protein. Muscarinic stimulation from parasympathetic neurons (vagus nerve) increases cytosolic calcium via DAG/PKC pathway. Ligand binding to GPR40 also results in increased cytosolic calcium, but the precise mechanism for this has not yet been determined.

1.4.2 Secretory products of the beta cell: IAPP

Islet amyloid polypeptide (IAPP) is the second most abundant secretory product of the pancreatic beta cells. Historically, IAPP has also been referred to as amylin because misfolded IAPP forms islet amyloid. This terminology can be confusing, thus the name IAPP has become more widely accepted. Transcription of IAPP is regulated and promoted similarly to insulin. IAPP is translated as a 67amino acid prohormone that is processed by PC1 and PC2 to a mature, 3.9kD, 37 amino acid peptide with a single disulfide bond between cysteine residues at position 2 and 7.[19] IAPP is trafficked alongside insulin and packaged within secretory vesicles.[20] Within these vesicles, the mature insulin and IAPP form a stable crystal structure (along with zinc ions) within the dense core of the granules. In the dense core there is between 25 and 50 times more insulin than IAPP.[18]

IAPP has several beneficial physiologic effects in the body including stimulating satiety, suppressing food intake, delayed gastric emptying, and inhibiting meal-associated glucagon secretion. [21-23]

1.5 The incretin effect

In 1906, a group of researchers at the University of Liverpool published the first work describing the treatment of diabetes mellitus in people using the extract of duodenal mucous membranes from pigs. In their work they described the effects of "secretin", which was shown to increase exocrine pancreatic secretion and had a glucose lowering effect on the body.[24,25] It was clear that compounds associated with the gut mucosa had a profound effect on the function of the pancreas and, prior to the discovery of insulin, it was hoped that these compounds could be useful in treating diabetes mellitus.[26] In 1932, a Belgian physician named Dr. Jean La Barre and his collaborator, Dr. Zunz, documented the hypoglycemic effect of duodenal extracts in normal

animals that was abolished in pancreatectomized animals. They named the substances responsible for this phenomena "incretins", a term still in use today.[27]

By the early 1960s, it was possible to measure plasma insulin in people. In 1964, what is now known as the incretin effect was first described by two separate groups.[28,29] The incretin effect is defined as the magnified release of insulin in response to a dose of glucose given enterally as compared to an equivalent dose given intravenously. The effect can be profound, accounting for as much as 60% of the increase in insulin after oral ingestion of glucose in a healthy adult human.[30,31] The hormone responsible for this response was unknown until 1970, when a new hormone named gastric inhibitory peptide was purified from duodenal extracts.[32] A second major incretin, glucagon-like polypeptide, was not described until 1980.[33] The incretin effect has been demonstrated in many species, including people, dogs, rodents, and cats.[34,35]

1.5.1 Incretin Hormones

The incretin effect described above is due principally to the effects of two hormones. Gastric inhibitory peptide (GIP) and glucagon-like polypeptide (GLP-1) are products of specialized enteroendocrine cells. GIP is produced by K-cells and GLP-1 is a product of L-cells. GLP-1, like glucagon, is a product of the proglucagon gene. In the alpha cells of the pancreatic islets, expression of prohormone convertase 2 (PC2) leads to production of glucagon and major pro-glucagon fragment (MPGF). MPGF appears to be biologically inactive. In intestinal L-cells, expression of PC1 leads to production of GLP-1, GLP-1, and glicentin. (Figure 1.4) The largest amount of GLP-1 is produced by L-cells in the small intestine and colon. There is some minor variability of location of these cells within the gut of different animals.[30,36] It has been shown in the cat that GLP-1 positive L-cells are found throughout the gut, but are concentrated in the ileum. GIP is produced within K-cells that are equally distributed throughout the feline gut.

Unlike what is observed in other species, K-cells and are not concentrated in the adoral portions of the gut in cats.[37]

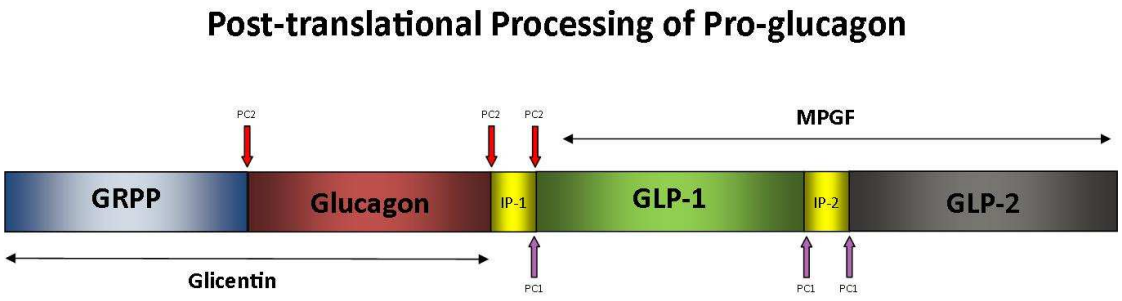

Figure 1.4: Proglucagon is differentially processed in the intestinal L-cells and the brain where PC1 is highly expressed. This results in the major products GLP-1, GLP-2, and glicentin. In the pancreatic alpha cells, PC2 is highly expressed. Here, the resulting products are glucagon, GRPP, and the major proglucagon fragment (MPGF)

GLP-1 has an extremely short half-life in the body (1-2 minutes in people) due to the effects of dipeptidyl peptidase-4 (DPP4).[38] DPP4 is ubiquitously expressed in the body and is found both free and tissue-bound forms. DPP4 is a serine protease enzyme that cleaves the two c-terminal amino acids from active GLP-1 (7-36), thereby generating the inactive metabolite GLP-1 (9-36). Of the total GLP-1 produced by the L-cells, only 25% reaches portal circulation and only 10% reaches the caudal vena cava and systemic circulation.[30]

Stimulation of K and L-cells occurs in response to nutrient exposure from luminal contents of the gut. The nutrients responsible for the strongest stimulation of these cells vary slightly between species. In the cat, unlike in people, release of GIP is highest in response to lipids and amino acids, but minimal to glucose. Conversely, GLP-1 release in cats was equally stimulated by glucose, amino acids, and lipids.[34] The response of GIP to lipids and amino acids was much more robust than that seen in people, whereas the response of GLP-1 to lipids and amino acids was much weaker than that seen in people.[39]

One potential interpretation of these findings is that this pattern of response is consistent with the natural history of the cat. As an obligate carnivore, the cat should be more responsive to lipids and amino acids and less responsive to simple carbohydrates. Studies completed in people have shown the incretin effect is blunted in people who are obese or Type 2 diabetic but this has not been investigated in cats.[40]

1.5.2 Effects of GLP-1: Insulin production and release

GLP-1 acts both directly and indirectly on pancreatic beta cells to potentiate acute phase insulin release. The effect of GLP-1 on vagal afferents elicits a vagal-vagal reflex and will produce immediate muscarinic stimulation of insulin release from the beta cells.[41-43] The GLP-1 receptor is a g-protein coupled receptor and ligand binding of the GLP-1r on beta cells induces

adenylyl cyclase, which in turn activates two parallel pathways by which GLP-1 potentiates acute insulin release. Increases in cytosolic cAMP inactivate the membrane-bound potassium ATPase and assists in depolarization of the cell membrane.[43] Additionally, the increasing cytosolic cAMP activates the EpacII "exchange protein activated by cyclic AMP-2".[43] EpacII directly inactivates the potassium ATPase pump and binds the ryanodine receptor on the endoplasmic reticulum, thereby increasing cytosolic calcium.[23] (Figure 1.5)

1.5.3 Effects of GLP-1: Beta cell sparing effects

Of particular interest in regards to therapeutic implications are the supportive effects GLP-1 exerts on the beta cells, generally characterized as anti-apoptotic, pro-proliferative, and anti-inflammatory.[44-52] GLP-1 has been shown to decrease the severity of the unfolded protein response during periods of endoplasmic reticulum stress in the beta cell.[53] GLP-1 is a potent promoter of the PDX-1 gene in the pancreatic beta cell.[49,54] The PDX-1 promoter is central to beta cell secretory, replicative, and survival mechanisms.[49,55-57] GLP-1 has been shown to confer anti-apoptotic protection against cytokine-induced and hyperglycemic apoptosis in both insulinoma cell lines and isolated human islets via activation of the AKT/PKB pathway.[46,51,58] (Figure 1.5)

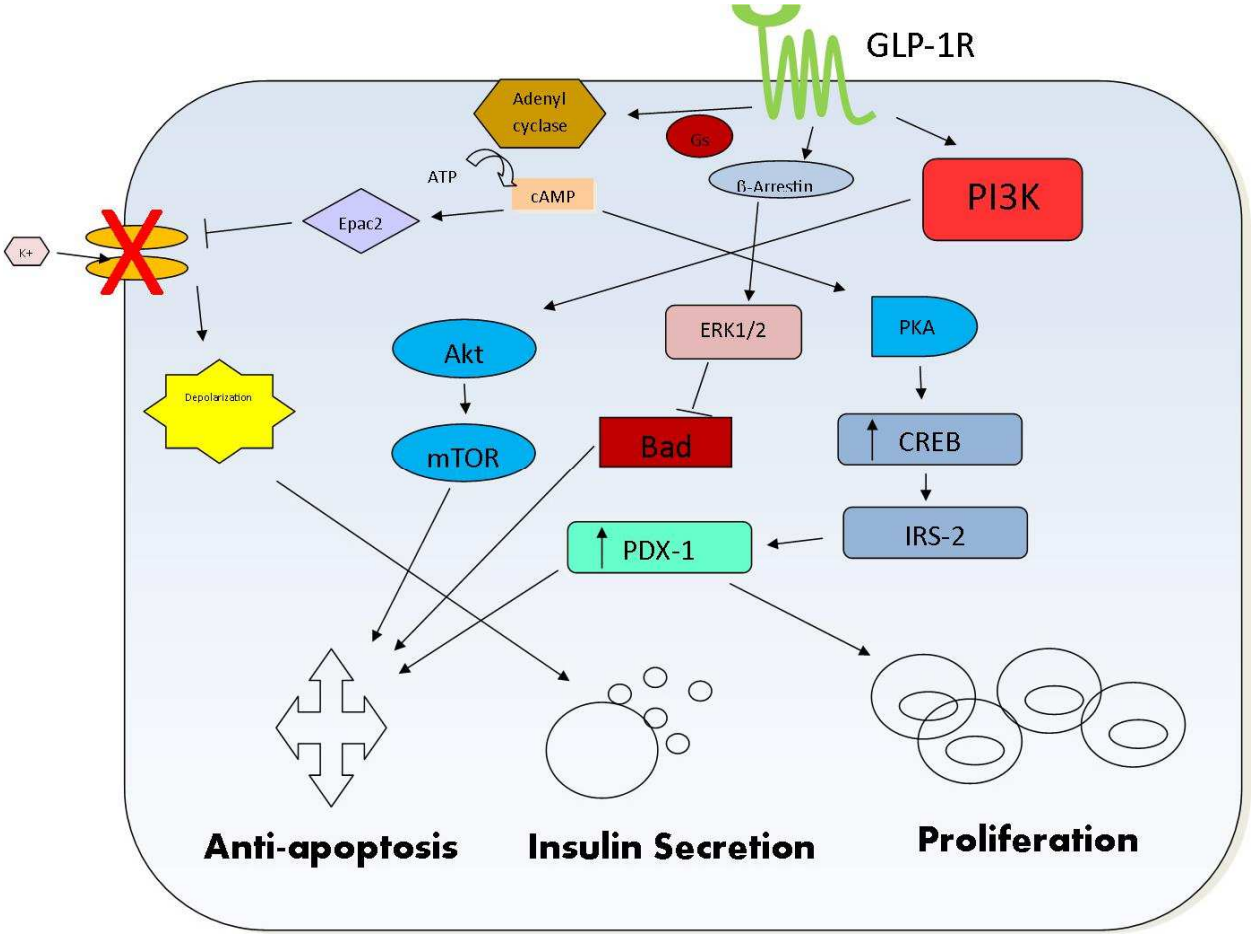

Figure 1.5: GLP-1 signaling in the pancreatic beta cell has three primary results. It is antiapoptotic via the Akt, ERK1/2, and CREB/PDX-1 pathways. Insulin secretion is enhanced by Epac2 closure of the potassium ATPase channels assisting in membrane depolarization. GLP-1 promotes proliferation primarily based on its promotion of PDX-1.

Pancreatic beta cells demonstrate remarkable plasticity. Although typically a long-lived cell type in healthy individuals, beta cell neogenesis, duplication, differentiation, and expansion are seen when insulin demands exceed the capacity of the current beta cell population.[59] GLP-1, via cAMP/PKA and MAPK, promotes beta cell replication and expansion through activation of cyclin D.[60] Partially pancreatectomized mice treated with a GLP-1r agonist maintained greater beta cell mass and better glucose tolerance as compared to control mice.[61] Pretreatment of mice with exendin-4 (a synthetic GLP-1 receptor agonist) prior to administration of streptozotocin resulted in significantly lower blood glucose and increased oral glucose tolerance, suggesting a beta cell sparing effect of GLP agonists.[47] This effect was lost on GLP-1R knockout mice. In streptozotocin treated newborn mice, GLP-1 stimulated beta cell neogenesis and led to a nearly normal glucose tolerant phenotype.[62] Goto-Kakizaki rats are born with limited beta cell mass and rapidly become insulin-dependent diabetics. Experiments have shown in juvenile Goto-Kakisaki rats that two months of treatment with GLP-1 and GLP-1 mimetics was associated with a significant reduction in blood glucose and expansion of beta cell mass.[63] Not only can new beta cells be generated by expansion of existing beta cells, but it is also apparent that pools of pluripotent stem cells within islets and ductal epithelium can be induced to differentiate into insulin producing beta cells under the influence of GLP-1.[64] Direct inhibition of apoptosis by GLP-1 and GLP-1 mimetics has been demonstrated in multiple cell lines.[46,47,58,65]

GLP-1 has been shown to have many activities in the body outside of its effects on the beta cell. In the brain, GLP-1 triggers satiety signals, promotes neural stimulation of insulin release via the vagal nerve and has been suggested to help inhibit the deposition of the amyloid-beta plaques typical of Alzheimer's patients.[66] In the heart, GLP-1 increases intracellular calcium and has positive inotropic and chronotropic effects.[31]

The effects of GIP appear to be more limited. GIP receptors are in the same family of G-protein coupled receptors as GLP-1R, and are found in the gut, pancreatic beta cells, brain, and

bone. Binding of GIP to its receptor results in an increase in cyclic AMP via activation of adenylyl cyclase. GIP has been shown to inhibit apoptosis by an increased expression of Bcl-2 and decreased expression of Bax in rodent insulinoma and isolated rodent islets.[31,38,67,68] GIP and GLP-1 together are responsible for the incretin effect in people.[30] However, recent studies in the cat suggest that the incretin effect in cats is mediated not by GIP, but by GLP-1 alone.[34] Whether GIP maintains the other positive effects on insulin promotion or in the role of preventing apoptotic stimuli has not been evaluated in the cat.

1.6 Mechanisms of Pancreatic Beta Cell Dysfunction

Our appreciation for the profound complexity of metabolic function and signaling in the pancreatic beta cell has continued to evolve as new receptors and metabolic pathways are discovered. It is clear that feline diabetes is a polygenic disease involving many different pathways that all ultimately lead to a state of glucose intolerance in the body. The most commonly investigated mechanisms include the relative oxidative state of the beta cell and the body as a whole, nutrient intake and relative nutrient load delivered to tissues, tissue sensitivity to hormones, disruption of normal cellular signaling in beta cells, alterations in beta cell autophagy, and alterations in peripheral insulin sensitivity due to comorbidities such as systemic inflammatory conditions and chronic endocrinopathies.

The function of the beta cell is to be responsive to changes in nutrient load and metabolic demands of the body. As discussed above, the beta cell is able to sense the nutrient load of the surrounding interstitial fluid (glucose and NEFAs) and provide a metabolically appropriate response in the form of the production and release of the precise amounts of insulin and IAPP required to maintain euglycemia and stimulate satiety while providing adequate nutrients to insulin sensitive tissues.

The most significant mechanisms for beta cell dysfunction, as they are currently understood, can be grouped into 5 general categories: gluco-lipotoxicity, inflammation, oxidative stress, amyloid accumulation, and endoplasmic reticulum stress. These categories are not mutually exclusive, and most pathways of dysfunction cross over the arbitrary definitions presented here. For instance, the stress of excessive nutrients (as discussed below) is closely associated with both increases in oxidative stress and inflammatory cytokine production. (Figure 1.6)

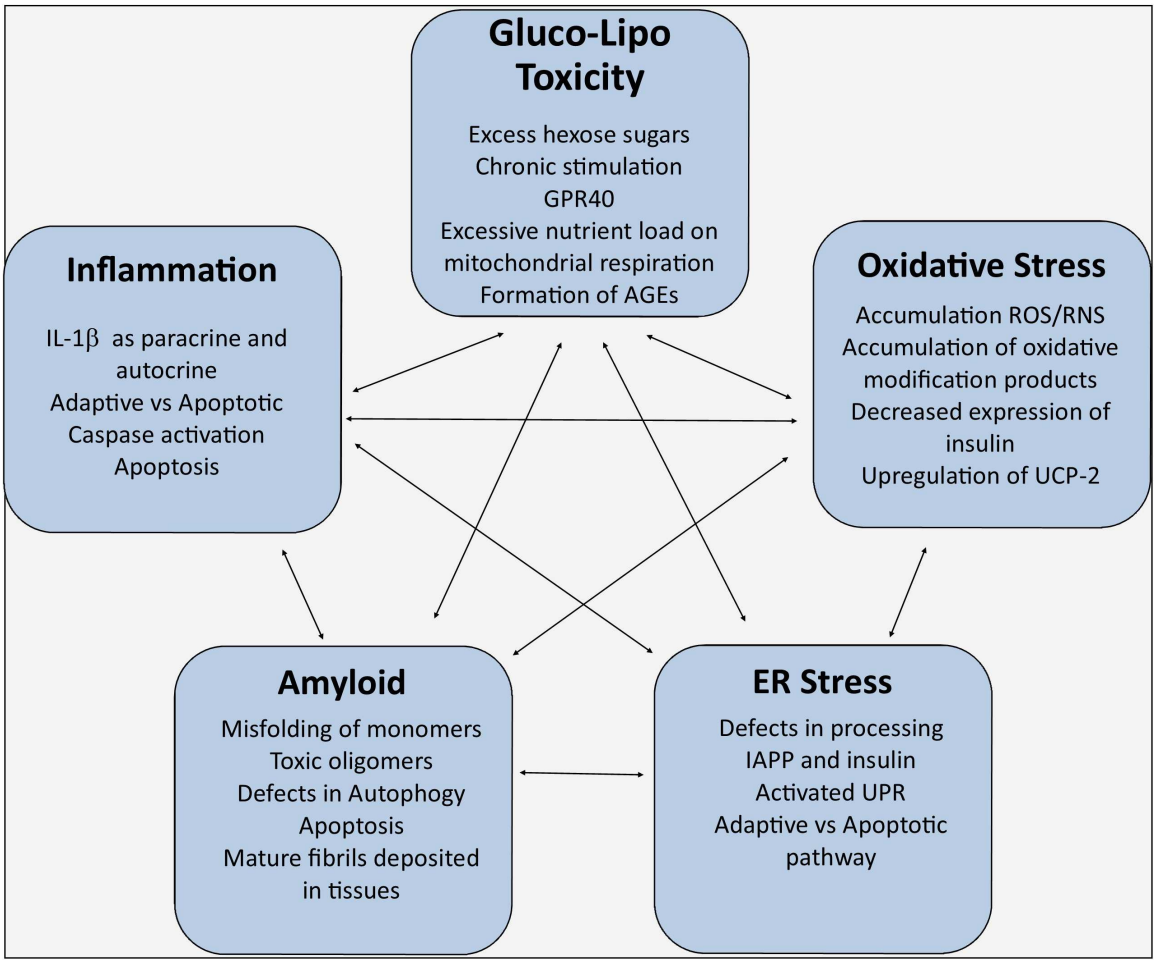

Figure 1.6: The interplay between the different causes of beta cell dysfunction is complex. Gluco-lipotoxicity is most likely the result of increased oxidative stressors on the beta cells but is also associated with increased inflammatory cytokine signaling. Interleukin-1β plays a central role in mediating islet inflammation and IL-1β produced by beta cells acts as both an autocrine and paracrine inflammatory mediator. Hyperglycemia alone is associated with oligomeric amyloid formation. Oxidative stress is both a cause and consequence of inflammation and amyloid production. The role of amyloid is still hotly debated, but it appears clear that oligomers of misfolded amyloid are associated with increased oxidative stress and inflammation.

1.6.1 Glucotoxicity

The phenomenon of glucotoxicity to the beta cell is well established.[69,70] Due to the high affinity nature of GLUT-2 transporters the beta cell is unable to reduce glucose influx and therefore can be exposed to extremely high amounts of glucose during periods of hyperglycemia. The most significant mechanism of glucotoxicity involves increases in oxygen free radicals secondary to increased metabolic rate and influx of hexose sugars into the beta cell.[71] The role of oxidative stress is discussed below. Although acute hyperglycemia is a powerful stimulant for insulin biogenesis and release, chronic hyperglycemia is associated with a decrease in insulin production and loss of PDX-1 and MafA activity.[72] Exposure to excessive glucose has been shown to decrease the transcription factor Pax6 in INS-1E cells. As discussed earlier, Pax6 is an important promoter for insulin transcription.[73]

Excessive glucose is also associated with increased production of carbohydrate adduct products on proteins known as advanced glycation end-products (AGEs). AGEs have been shown to be increased in people with decreased insulin sensitivity and in diabetic people.[74] In rodent insulinoma cell lines and in freshly isolated rodent islets, exposure to AGEs has been associated with the production of ROS, increased rates of apoptosis, and decreased insulin gene transcription.[75-77]

The toxic effects of hyperglycemia on the beta cell have been established *in vivo* in the cat. Cats experienced profound loss of islet architecture and beta cell mass during a 10-day hyperglycemic clamp as compared to saline controls.[78] During the 10 day study period, blood glucose concentrations were clamped at 25-30mmol/l, a concentration consistent with that of an uncontrolled diabetic. Studies investigating changes in islets under more moderate blood glucose conditions, more closely approximating the developing stages of diabetes, are needed to

determine the importance of hyperglycemia alone on development of beta cell loss and islet destruction.

1.6.2 Lipotoxicity and the role of GPR-40

Dyslipidemia characterized by excessive non-esterified fatty acids (NEFAs), increased plasma cholesterol, and abnormalities in lipoprotein content are common sequelae of both "metabolic syndrome" and type 2 diabetes in people.[79,80] The pancreatic beta cell appears to be particularly sensitive to the toxic effects of the NEFA palmitic acid.[81,82] Insulin resistance and feline diabetes are associated with a dyslipidemia in cats as well.[83]

Beta cells express a membrane receptor in the g-protein class commonly known as the fatty acid receptor or GPR-40.[84] Although this receptor is ubiquitously expressed, there is particularly high expression associated with the beta cells in mice and people.[85-87] It is unknown if this receptor is expressed in feline islets.

Various free (non-esterified) long-chain fatty acids are ligands for this receptor.[88] In health, binding of NEFAs to GPR-40 potentiates insulin secretion via PLC- mediated increases in cytosolic calcium.[87] However, in the face of chronic nutrient excess in the form of hyperlipidemia, particularly excess palmitic acid, binding of GPR-40 is associated with pro-apoptotic stress signaling via activation of JNK and MAPK.[89, 90]

Although cats do experience a modest dyslipidemia associated with obesity and insulin resistance, this is minor in comparison to the degree of dyslipidemia that occurs in people.[79,91] Additionally, cats do not suffer the chronic cardiovascular complications commonly seen in obese or diabetic humans such as atherosclerotic plaques and hypertension. Chronic obesity in cats is associated with a more severe dyslipidemia characterized by a VLDL triglyceride fraction

increase of 500% as compared to lean control cats. NEFAs were also increased in the long-term obese cat, but the species of NEFA and degree of saturation were not investigated.[83,92] In another study involving a 10-day hyperlipidemic clamp in which circulating palmitic acid was increased, there was little to no change in beta cell function or morphology.[93] This was in stark contrast to cats subjected to a 10-day hyperglycemic clamp, during which time profound loss of beta cell function and mass were observed.[78] Taken together, these results suggest that feline beta cells are not as sensitive to lipotoxicity as human beta cells.

1.6.3 Oxidative Stress

1.6.3.1 Reactive Oxygen and Nitrogen Species in Health

Reactive Oxygen Species (ROS) are generated via several pathways. The majority of superoxide radicals are the result of mitochondrial respiration and production of ATP via the electron transport chain. Under a normal metabolic state, superoxide is promptly detoxified by the enzyme superoxide dismutase (SOD), a ubiquitous enzyme that exists both within the mitochondria (SOD-2) and free in the cytosol (SOD-1). SOD enzymes utilize the oxidative state of metals such as manganese, zinc, or copper to facilitate the addition of protons to the superoxide molecule and the creation of hydrogen peroxide.

Hydrogen peroxide can be further reduced to oxygen and water by the actions of glutathione peroxidase using NADPH as electron donor. Alternatively, hydrogen peroxide may also spontaneously react with various metals via the Fenton reaction and produce free hydroxyl radicals. These highly reactive molecules can react with lipids and generate lipid peroxides, proteins to create protein carbonyls, or can react with nucleic acids creating unstable modifications to DNA.

Reactive nitrogen species are ubiquitous and important biological signaling molecules.[94] The action of specific nitric oxide synthase enzymes metabolize arginine to citrulline and create nitric oxide. Nitric oxide is a commonly employed second messenger in smooth muscle causing relation of muscle fibers.

It is important to realize that exposure to free radicals such as reactive oxygen and nitrogen species is not always detrimental to the cell. Oxygen free radicals, hydrogen peroxide, and nitric oxide all contribute to the overall REDOX state of the cell and are known to serve as second messengers or signaling molecules.[94] Problems develop when there is an imbalance in REDOX potential producing an excess of oxygen radicals or a lack of antioxidant protection.

1.6.3.2 Reactive Oxygen and Nitrogen Species in Disease

Because many oxidative modifications are relatively stable, it is common to use their presence as an indicator of oxidative stress or damage to the cell. Reactive oxygen species interacting with membrane lipids can generate oxidized lipids. The most commonly quantified markers of oxidative modification of lipids are malondialdehyde as measured by the thiobarbituric acid reactive substance test (TBARS) or hydroxynonenal (4-HNE). Oxidative modification of proteins can be the result of direct amino acid oxidation, lipid aldehyde adducts, or the result of reactive nitrogen species (RNS) interacting with certain amino acid side chains resulting in a variety of covalent modifications including 3-nitrotyrosine (3NT). In the nucleus, oxygen free radicals interact with nucleic acids and can produce adducts, single and double strand breaks, or relatively benign modification of individual nucleic acids. 8-hydroxyguanosine (8-OHG) is routinely used as a marker of oxidative damage to nucleic acids. Techniques using directed antibodies can be used to identify and quantify oxidative modifications such as 4-HNE, 3-NT, and 8-OHG.

1.6.3.3 ROS and RNS in Beta Cell Dysfunction

The exact role of oxidative stress and oxidative stress induced damage in beta cell function and dysfunction is not clear. In mice and people it has been shown that beta cells, as compared to other metabolically active tissues, have a fairly weak antioxidant capacity.[95-98] This peculiarity has led to several theories regarding the role of oxidative stress and beta cell dysfunction.

Beta cells are intended to respond rapidly to ambient nutrition in the intercellular space. The beta cell is somewhat different than other cells in that the glut-2 transporter expressed by beta cell does not allow for regulation of nutrient influx into the cell. Therefore, the metabolic rate of the beta cell is set not by its needs but by the availability of nutrients (namely glucose). As described above, glucose (and other hexose sugars) are rapidly metabolized and shuttled into mitochondrial respiration for the generation of ATP (a process required to induce insulin release in response to an increased ATP:ADP ratio in the cell). However, when the electron transport chain becomes overwhelmed, or when ATP content of the cell is exceedingly high, the proton gradient across the mitochondrial membrane can be "short-circuited" and allowed to leak. This will slow the production of ATP, but also generates an increase in free radical generation. This short circuit is largely due to the expression and activity of uncoupling protein 2 (UCP-2), which has been shown to be up-regulated in beta cells in response to nutrient excess.[99,100]

Alterations in oxidative signaling or increases in oxidative stressors on the beta cell will induce a wide range of response and adaptive mechanisms including down-regulation of beta cell secretory products, up-regulation of NF-κB, and activation of JNK.[95,101-104] Experiments in freshly isolated rat islets exposed to oxidative stress have demonstrated a rapid reduction in insulin gene promotion and nuclear export of PDX-1.[95,105] Induction of the NF-κB pathway is complex and

can be associated with a cytoprotective and pro-proliferative response in the beta cell. However, aberrant signaling resulting from excessive or chronic stimulation of NF-κB is associated with increased pro-inflammatory gene products (such as interleukin-1), induction of inducible nitrate synthase (iNOS), and ultimately induction of apoptosis. Activation of c-Jun N-terminal kinases by excessive ROS formation is also associated with a decline in beta cell function and induction of beta cell apoptosis.[103]

There have been no studies to date investigating the precise role of oxidative stress on the feline pancreatic beta cell. Additionally, there have not been any studies describing the antioxidant capacity of the feline beta cell to determine if it is relatively deficient in antioxidant capacity similar to what has been described in people and rodents. Although data is lacking regarding the local pancreatic antioxidant capacity in the cat, the evidence is mixed regarding whether cats experience increased levels of whole body oxidative stress similar to that seen in both obese and Type 2 diabetic people.[6,83,106-110]

1.6.4 Amyloid

Pancreatic amyloid protein is composed of aggregated misfolded monomers of IAPP. Amyloid deposits within pancreatic islets are a common feature of both T2D in people and FDM. Estimates suggest deposits of amyloid are found in greater than 90% of people and cats with diabetes mellitus.[111-116] Additionally, islet amyloid can occasionally be found in apparently healthy adult and geriatric people and cats.

The amyloidogenic potential of IAPP to misfold is largely determined by the amino acid sequence, but is also influenced by protein concentration, pH, associated stabilizing cations, and stabilizing chaperone proteins.[18,117-120] Not all mammalian native IAPP peptides are able to

misfold into amyloid protein. The peptide sequence between residues 25 and 29 appear to be particularly important, although sequence at the n-terminus of the peptide appear to also play a role.[18] (Figure 1.7) Only people, non-human primates, and cats are known to spontaneously form pancreatic amyloid. Mouse and rat IAPP is unable to form amyloid. It is believed that the inclusion of multiple proline residues between residues 25 and 29 in rodent IAPP introduces rigidity to the tertiary structure that will not allow folding and formation of the quaternary fibrillar structure.

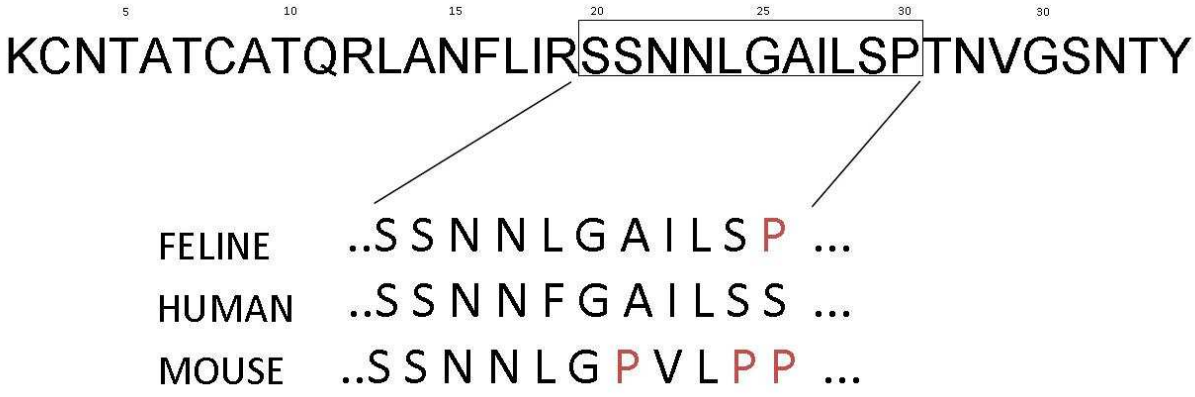

Figure 1.7: The putative sequence of IAPP protein believed to be responsible for the protein's ability to misfold is contained between residues 20-29. The inclusion of several proline residues introduces rigidity to the tertiary structure of the peptide that inhibit misfolding. Human and feline IAPP sequences are highly homologous.

Under normal conditions, IAPP monomers remain stable within the secretory granules. This stability is believed to be related to vesicular pH, crystal structure of insulin, and the presence of large quantities of Zn ions (millimolar concentrations within the vesicles).[121,122]

Under certain conditions, IAPP molecules are able to misfold and will spontaneously form small-order multimers. There is increasing evidence that these small oligomeric species of amyloid are quite toxic to the beta cell.[123-127] The presence of oligomeric amyloid has been confirmed in beta cells of diabetic and non-diabetic people and in rodents transgenic for human IAPP.[126,128] There are several hypotheses as to the nature of oligomeric amyloid toxicity to the beta cell. Oligomeric amyloid is capable of inserting itself into lipid bilayers, creating pores and compromising the integrity of the membrane.[125] The creation of such pores in the ER membrane, secretory vesicles, lysosomes, phagolysosomes, or cell membrane can have rapid and terminal effects on the cell. Fresh human islets cultured in media seeded with supraphysiologic amounts of human IAPP had dramatic changes in morphology, increased apoptosis, and decreased insulin release.[127]

IAPP misfolding and polymerization can propagate if not cleared by the cell, and extremely large and long fibrils of amyloid are formed.[18] Fibrillar amyloid is deposited in the extracellular space and it is the collections of fibrillar amyloid that are visible microscopically.

The presence of fibrillar amyloid in the diabetic and glucose competent cat is well documented. What remains unknown is whether feline IAPP forms oligomers and whether these smaller misfolded proteins are associated with beta cell dysfunction in the cat as has been suggested in people.

1.6.5 Endoplasmic reticulum stress

Post-translational processing of insulin and IAPP begins in the endoplasmic reticulum of beta cells. Beta cells must remain highly labile and responsive to insulin demand and as such the

rate of synthesis of the beta cell varies hour to hour. This rapid translation and variable demand makes the beta cells particular sensitive to accumulation of misfolded or incompletely folded protein in the ER. The association of ER stress and beta cell dysfunction in people and rodents is well described in the literature.[129-134] The unfolded protein response (UPR) is a mechanism by which cells are able to adapt to the volume of misfolded or unfolded proteins in the ER. The effects of UPR activation include reduction of protein transcription and promotion and recruitment of chaperone proteins. The UPR is highly adaptive and is largely regulated by three key proteins forming three canonical branches: protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6).[135] PERK, IRE1 and ATF6 are all ER trans-membrane proteins and activation of these proteins is regulated primarily by association of the endoplasmic domain of the proteins with the chaperone protein Grp78 (also known as BiP). Recruitment of Grp78 away from PERK, IRE1, or ATF6 during periods of high protein production or accumulation of misfolding proteins allows phosphorylation and dimerization of IRE1 and PERK and phosphorylation of ATF6. (Figure 1.8)

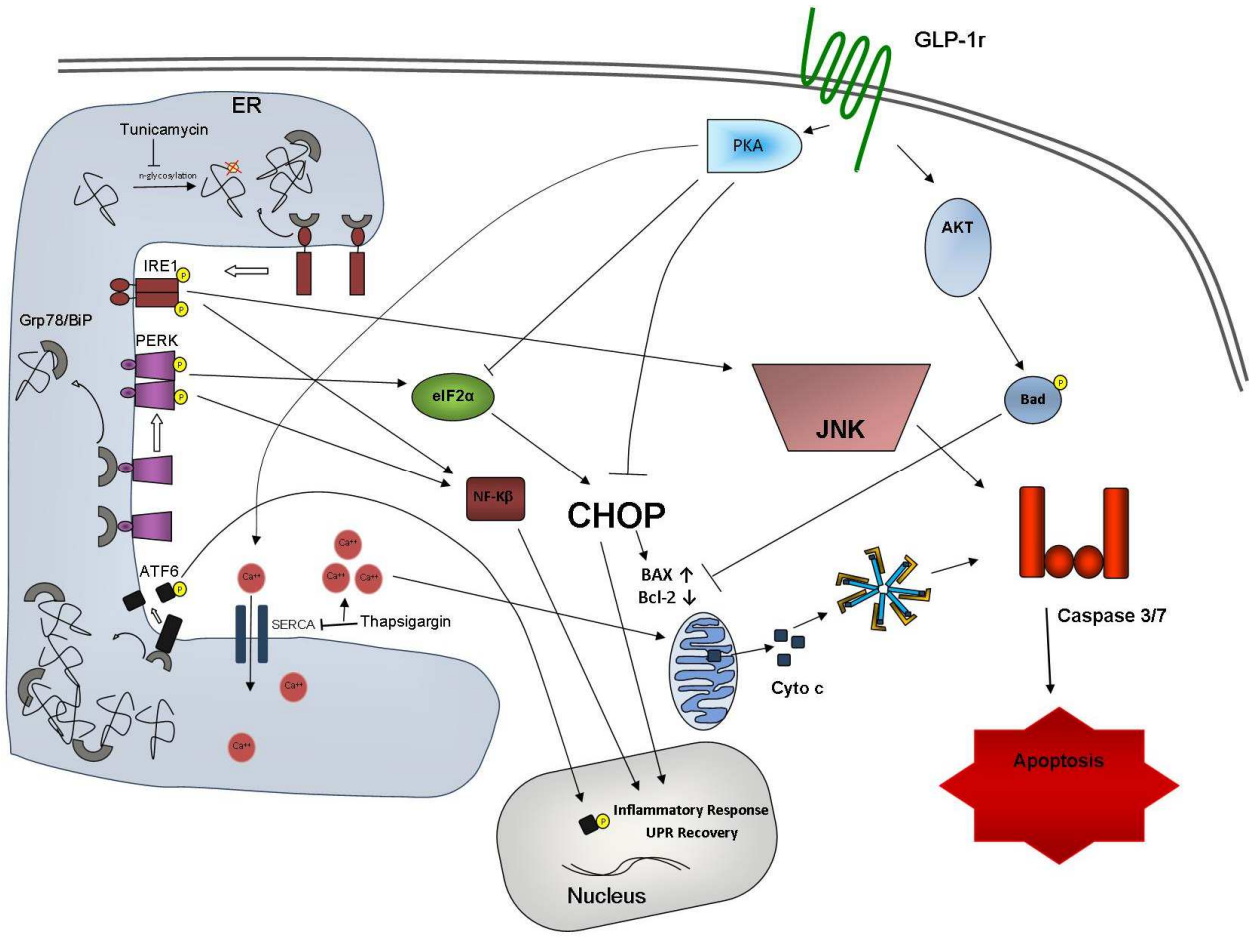

Figure 1.8: There are three canonical branches of the UPR. Accumulation of misfolded or unfolded proteins leads to disassociation of GRP78 from UPR mediating proteins, allowing dimerization and phosphorylation of IRE1 and PERK and phosphorylation of ATF6. IRE1 activation will in turn up-regulate the activity of XBP1s (not shown) which serves as a transcription factor to promote chaperone protein production (such as BiP). Excessive activation of IRE1 also activates the JNK pathway leading to apoptosis. PERK phosphorylates eIF2α which up regulates the activity of CHOP. CHOP functions include induction of mitochondrial stress (and activation of the intrinsic pathway to apoptosis) and serving as a transcriptional regulator of several pro-inflammatory and pro-apoptotic products. ATF6 phosphorylation allows for translocation of the cytoplasmic domain of the protein to the nucleus where it serves as a

transcription factor for, among other things, several chaperone and UPR-related proteins. Thapsigargin inhibits the function of the sarco/endoplasmic reticulum calcium ATPase (SERCA) pump, thereby depleting ER calcium stores. Decreased ER calcium causes ER swelling and promotes misfolding while increasing cytoplasmic calcium leads to activation of the intrinsic apoptotic pathway. Tunicamycin inhibits n-glycosylation of proteins, leading to misfolding and accumulation of these proteins within the ER. GLP-1r signaling is anti-apoptotic via several mechanisms: direct inhibition of eIF2α, activation of the SERCA pump, inhibition of CHOP, and inhibiting pro-apoptotic Bcl-2 family proteins via phosphorylation of Bad.

The primary downstream effects of PERK activation are mediated through eukaryotic translation initiation factor 2α (eIF2α). eIF2α in turn functions to inhibit global protein transcription and relieve workload of the ER. Excessive activation of eIF2α will also activate C/EBP homologous protein (CHOP) which is a potent transcription factor for various inflammatory and promotes pro-apoptotic pathways.

Under mild ER stress, IRE1 phosphorylation results in ribonuclease activity of the cytoplasmic domain and cleavage of the X-box binding protein-1 (XBP1) mRNA, thereby generating an alternative, active transcript. This spliced XBP1 in turn is a key promoter of chaperone proteins and phospholipid biosynthesis. Excessive stimulation of IRE1 will also result in activation of cJun n-Terminal kinase (JNK) that will result in caspase activation and a pro-apoptotic pathway. IRE1 also promotes IκB kinase (IKK) degradation and activation of nuclear factor κB (NFκB) which, as discussed later, can be both adaptive and mal-adaptive to the beta cell.

The third major canonical branch is mediated through the translocation of the phosphorylated cytoplasmic domain of ATF6 which serves as a potent regulator of multiple ER stress associated products such as chaperone proteins and cAMP response elements. As with the other branches, this regulation is adaptive under normal stimulation, but excessive stimulation will result in a pro-inflammatory, pro-apoptotic result.

The negative effects of prolonged activation of the UPC in beta cells are well documented and, as mentioned in section 1.5.3, activation of the GLP-1 receptor has been shown to modify the beta cell response to ER stress.[53,136-139] In both *in vivo* and *in vitro* experiments in rodents as well as cell culture experiments using human tissues treatment with GLP-1 or GLP-1 mimetics resulted in attenuation of the UPC in beta cells resulting in improved secretory capacity,

decreased rate of apoptosis, and down-regulation of components of the UPR after treatment with GLP-1 or GLP-1 mimetics.

1.6.6 Inflammation

The term "insulinitis" is used to describe inflammation associated with pancreatic islets. In the case of human type-1 diabetics, inflammation in response to immune-mediated signals is characterized by cellular infiltrates and a cytotoxic immune response directed against the beta cells. In Type 2 diabetic people, there is an increasing body of evidence supporting the role of inflammatory cytokine involvement in beta cell dysfunction and induction of apoptosis.[140-142] The insulinitis hypothesis centers around the pro-inflammatory cytokine IL-1β. Pancreatic beta cells express both IL-1β and the IL-1β receptor.[141,143] Multiple experiments originating from the same research group have demonstrated that increased glucose concentrations are associated with up-regulation of IL-1β and the autocrine and paracrine effects of IL-1β are major contributors to beta cell loss of function and apoptosis.[143-146] The importance of endogenously produced IL-1β is highlighted by the finding that the addition of an IL-1β receptor antagonist which protected beta cells from apoptosis when exposed to high glucose concentrations in culture.[143] Exposure of isolated rodent and human islet cells to IL-1β alone is also well described to induce beta cell apoptosis.

The role of IL-1β in progression of beta cell dysfunction is also suggested by several clinical trials using the IL-1R antagonist anakinra (Kineret®) which demonstrated utility of the drug by improving insulin responsiveness to glucose in pre-diabetic and Type 2 diabetic people.[147-150] These clinical findings are consistent with experiments using isolated human islets

and rat beta cells in which IL-1β receptor blockade with anakinra protects cells from pro-inflammatory cytokine induced necrosis and apoptosis.

The insulinitis hypothesis and the role of IL-1β is not universally accepted and there are conflicting experiments in the literature. Other research groups have been unable to detect IL-1β production in rat islets or in islets from pre-diabetic sand rats.[151] In a 2005 paper, islets harvested from non-diabetic human donors were cultured under high and low glucose concentrations, with and without IL-1R antagonists.[152] Islets were also harvested from Type 2 diabetic people and expression of IL-1β and NFκB was measured. The investigators concluded that the lack of correlation of IL-1β or NFκB to any changes associated with hyperglycemia or diabetes made it "unlikely that locally produced IL-1β is an important mediator of glucotoxicity to human islets and argues against the IL-1β/NFκB pathway as a common mediator for beta cell death in type 1 and type 2 diabetes."[152] Furthermore, the authors of a recent study using mouse islets concluded that beta cell apoptosis secondary to increased glucose or ER stress did not involve the NLRP3 inflammasome (and consequently IL-1β), and IL-1β knockout conferred no protection in response to glucotoxicity.[153]

There is no singular reason for such startling contradictions in the literature regarding the role of IL-1β in beta cell dysfunction. Undoubtedly part of the answer lies in the complexity of inflammatory cytokine signaling and beta cell response to acute and chronic nutritional changes. Minor stimulation of IL-1β, by activation of NF-κB, will promote pro-survival, pro-proliferative, and anti-apoptotic stimuli whereas chronic or excessive activation of NF-κB by IL-1β will drive signaling along a pro-apoptotic path.

The concentration of IL-1β that pancreatic beta cells are typically exposed to *in vivo* is not known. The IL-1 receptor is a high-affinity receptor and presumably biologic effects will be seen at doses well below the disassociation constant of the receptor.[141,154] Freshly isolated human

islets were reported by one group to produce a mean of 4.0pg/ml IL-1β after culture for 48 hours under baseline glucose concentrations.[155] This is in stark contrast to the 500-1000 fold higher concentrations (2-4ng/ml) of IL-1β used to induce beta cell apoptosis in other experiments.[143,156] There also appears to be a species difference in the susceptibility of pancreatic beta cells to the negative effects of IL-1β, with mice being relatively resistant to the effects while cows and rats are relatively sensitive and human islets have been reported as both very sensitive and very resistant to the effects of IL-1β.[154,157]

The duality of IL-1β signaling on beta cells was recently described in experiments using rat beta cells and freshly isolated human islets. In both rat and human tissues, exposure to low concentrations of IL-1β (10-100pg/ml) enhanced insulin production whereas exposure to high concentrations of IL-1β (20,000pg/ml) caused a marked decrease in insulin production.[158]

The role of inflammatory cytokine expression in feline beta cells has not been fully explored. It has been documented that feline beta cells express the IL-1β gene and that IL-1β mRNA increases after challenge with LPS in freshly isolated feline islet-like clusters.[159] However, in a separate study of cats after a 10 day hyperglycemic clamp, inflammatory cytokine (IL-1β, TNF-α, IL-6, IL-8, and monocyte chemoattractant protein-1) mRNA expression in isolated islet-like clusters remained unchanged despite a 4-fold decrease in insulin mRNA and profound overall loss of beta cell mass confirmed histologically.[78] Further work is needed to determine the relative sensitivity of feline beta cells to IL-1β.

1.7 In vitro methods to study beta cell function

Because of the complex nature of the disease, studying diabetes mellitus *in vivo* is exceptionally complicated. As has been outlined, adiposity, local and systemic inflammation,

peripheral insulin sensitivity, diet, background genetics, and redox state have all been shown to have a role in the development of clinical feline diabetes. Beta cell function and dysfunction is often inferred by evaluating the changes in secretory patterns of the beta cells (e.g. alterations of plasma insulin or IAPP), clinical signs (e.g. glycemic status or change in body weight), or in post-mortem evaluation of tissues taken after disease state is achieved. In vitro experiments using isolated islet tissue clearly have benefit because they allow for greater control and investigation of specific pathways involved in disease development unique to the environment of the pancreatic islet.

In vitro study of disease pathogenesis is complicated by the lack of fully physiologically competent cell lines that can be immortalized in culture. The majority of cell lines used in diabetes research are rodent insulinoma lines produced as either transgenic or radiation induced cells (including INS-1, RINm5F, and MIN6). There is at least one human insulinoma cell line cultured from metastatic carcinoma cells collected from a single patient (CM cell line). Unfortunately, none of the available cell lines are physiologically normal in their ability to produce insulin, their response to glucose, or their expression of glucose transporters. There are currently no immortalized cell lines from the cat available for study.

The exocrine pancreas is rich in digestive enzymes such as lipase and amylase, which can be very damaging and detrimental to tissue stability once in culture and thus represent a significant hurdle to using whole-organ explant or transplant for *in vitro* study. Intact pancreatic islets provide a good alternative because, although technically challenging to isolate, they allow a complete separation from the exocrine pancreatic tissue while providing a complex environment of multiple cell types, each with significant paracrine effects on its surrounding cells. Maintaining cell-cell interactions and paracrine signaling allows for a more physiologic response of the beta cells to manipulation.

Primary cell culture from people or rodents is commonly used for in vitro study of disease mechanisms. Techniques for the liberation and purification of intact islets or individual beta cells are well established for these species. Most protocols rely on collagenase-based tissue digestion to liberate islets and, if desired, trypsin dispersion of the islet to individual cells to isolate the beta cells. Once isolated, islet tissue can either be stained by the zinc chelating dye dithizone (which turns zinc-rich beta cells a bright red color) and hand-picked for purity, or can be separated using density gradient centrifugation.[160] The latter is the method most commonly utilized in rodents and pigs.[161-164]

There have been two attempts documented in the literature to produce purified feline islets for laboratory study or tissue transplant. In the first published experiments, feline pancreata from cadaveric donor cats were subjected to collagenase digestion and the resulting digestate was cultured in enriched media.[165] Although there was some manual selection for "exocrine free" islet-like clusters, significant exocrine tissue still remained. The yield of islet clusters free of exocrine tissue was estimated to be 300-500 islets per cat. Additionally, the whole pancreatic digestate was allowed to rest in culture media for a period of 1-9 days. The estimated percentage of insulin positive cells in this milieu was never higher than 5%. Islets of cats should contain at least 60% beta cells, and so these results suggest that either the resulting digestate contained significant amounts of exocrine tissue or isolation resulted in the loss of more than 80% of islet beta cells. Investigators in this study also noted that although the "islet like clusters (ICCs)" attained a more uniform shape over the 9-day period, there was a decreasing amount of insulin positive tissue and much of the remaining tissue was proliferating ductal epithelium. ICCs transplanted into recipient cats underwent rapid rejection and none of the cats experienced more than 12 days of normoglycemia and life expectancy did not exceed 27 days.

This study had several limitations, the most significant of which was the lack of purified islets that retained some degree of normal secretory function. The failure of collagenase

digestion to yield large quantities of islets clean of exocrine tissue was briefly investigated. Electron microscopic findings suggested that most of the islet tissue was closely associated with the surrounding acinar tissue, with little to no collagenous connective tissue between them. It was postulated that the lack of connective tissue was the most likely reason for a failure of collagenase digestion to produce clean islet tissue. Unfortunately, in the unpurified tissue (whole digestate) the authors failed to yield any number of apparently pure or functional islets. The investigators transplanted between 3,500 and 23,000 of these impure "ICCs" into the sub-retinal space of cats. It is therefore not surprising that there was an unimpressive clinical response and rapid failure of the tissue grafts.

The second set of documented experiments to produce purified islets from the cat met with similar success in regards to yield of purified islets.[166] In this experiment, six collagenase-based methods for islet isolation were compared. The differences between protocols included second course collagenase digestion, ficoll gradient purification, and use of a second enzyme (Accutase®) to attempt to disperse islets more efficiently. Digestion with collagenase yielded large quantities of islets still embedded within exocrine tissue with approximately 2% of islets identified as "free". None of the six methods produced any significant increase over another regarding volume of purified islets. Islets harvested in these experiments were not cultured and their secretory capacity was not evaluated.

1.8 Treatment of feline diabetes mellitus

1.8.1 Insulin

Standard therapy of feline diabetes has two main arms, both of which focus on returning the patient to euglycemia. First, insulin hormone must be replaced to lower blood glucose. This is typically accomplished via administration of parenteral insulin. Secondly, insulin sensitivity of

tissues must be improved to reduce the demand on an already failing beta cell population. To this end, weight loss to restore insulin sensitivity and insulin sensitizing medications can be employed.

It is commonly accepted that most newly diagnosed, hyperglycemic diabetic cats (similar to Type 2 diabetic people) have some degree of residual beta cell function remaining at the time of diagnosis. For many of the reasons discussed above, loss of beta cell function and development of peripheral insulin resistance is a gradual process. This fact has led to significant interest directed towards therapies that can help restore functional beta cell mass and improve peripheral insulin resistance. The goal is to return the patient to euglycemia without the need for parenteral insulin, a state termed "diabetic remission." This definition acknowledges that while the patient is not considered "normal" or permanently glucose competent, they do not currently require insulin therapy.

Aggressive insulin therapy and tight regulation of euglycemia is associated with an overall diabetic remission rate of 64%.[167] It is believed that the reduced glycemic load reverses the effects of glucotoxicity and allows the beta cells of the pancreas to recover. The concept that beta cell "recovery" is possible after extended hyperglycemia and documented beta cell dysfunction was demonstrated in a 2013 study.[168] In this experiment, cats were clamped at a high serum glucose concentration (30mMol) for a period of 42 days. During this time, endogenous insulin production was initially increased but quickly decreased to the lower limit of detection for the duration of the study. Biopsy samples taken at the end of the hyperglycemic period confirmed wide-spread destruction of islet tissue in all cats and loss of insulin-positive beta cells positive for insulin using immunohistochemical stains. Six of 16 cats were treated with insulin during the hyperglycemic clamp to avoid ketoacidosis. However, once the infusion of glucose was discontinued, 10 of 16 cats required no parenteral insulin to maintain euglycemia and insulin

therapy was discontinued in the remaining 6 cats within the following two weeks. After a 21-day "recovery" period, all cats were able to maintain euglycemia without any medications.

1.8.2 Incretin mimetics

Significant potential exists for the treatment of feline diabetes mellitus with incretin mimetics.[169] As previously outlined, incretins and various mimetic drugs (most notably exendin-4) have been documented to induce satiety, promote weight loss, increase beta cell responsiveness to glucose, rescue beta cells from oxidative damage, and restore depleted beta cell mass in rodents and in people. The theoretical benefits in the cat cannot be overstated if each of these findings translated clinically to treatment of the feline diabetic.

There are two fundamental approaches to incretin therapy. One approach is to inhibit degradation of GLP-1 and GIP by inhibiting the DPP-4 enzyme. The second approach utilizes synthetic compounds with similar affinity for the GLP-1 receptor but that are resistant to DPP-4 activity. (Figure 1.9) To date, two GLP-1 mimetics (exenatide and liraglutide) and two DPP-4 inhibitors (sitagliptin and NVP-DPP728) have been investigated in cats. Exenatide short acting (Byetta®, Amylin Pharma) has been evaluated by two research groups and in both cases it was found to have a dose dependent insulinotropic effects in healthy cats with no reported severe adverse events associated with the drug.[170, 171] Only one study describes chronic administration of exenatide and the drug was not associated with adverse events, although weight loss was documented in all cats receiving drug.[171]

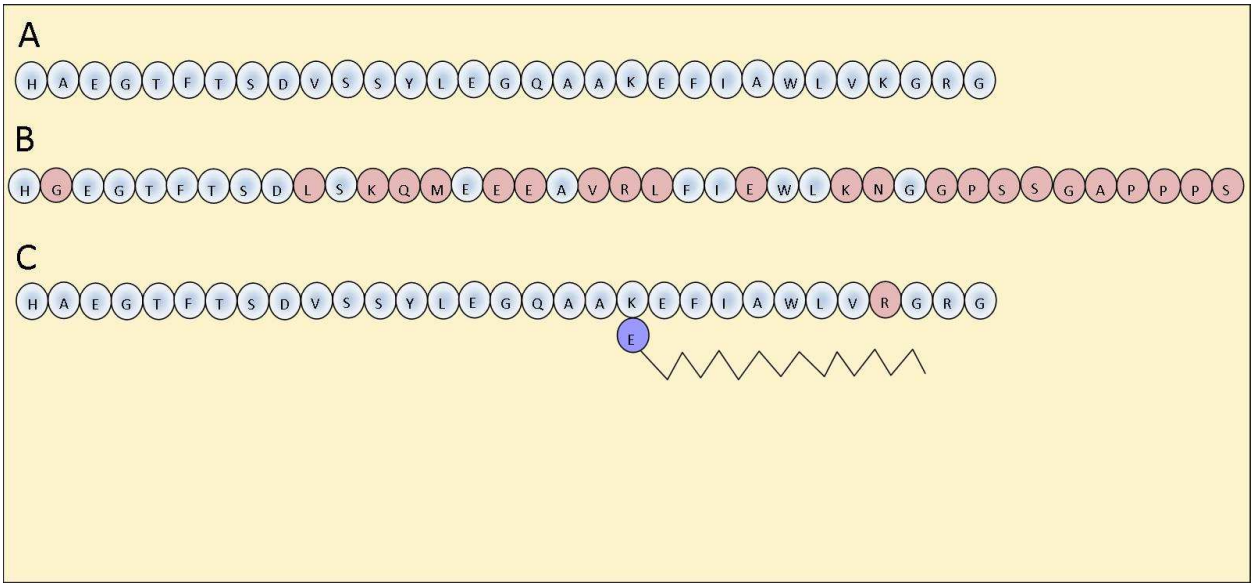

Figure 1.9: (A) Sequence for human GLP-1. The sequence for feline GLP-1 has not been determined. (B) Exendin-4 (exenatide) shares only 35% homology with GLP-1 but the substitution at position 2 of glycine for alanine confers resistance to DPP4 degradation. (C) Liraglutide shares near 100% sequence homology to native GLP-1, but the addition of a 16-carbon fatty acid side chain allows for binding to albumin and protects the hormone from degradation by DPP4.

There are two recent publications evaluating the pharmacokinetics and pharmacodynamics of long-acting exenatide (Bydureon®, Amylin Pharma).[172,173] Exenatide extended release was documented to have insulinotropic effects at least three weeks after initial injection with a peak in plasma concentration of the drug seen at four weeks post injection with no adverse events noted.[173] In another study, exenatide long-acting was compared to exenatide short-acting. In this study, exenatide short acting was associated with a more robust effect on insulin release. Exenatide short-acting was associated with an increased insulin area under the curve of between 320 and 540% as compared to 95-178% for exenatide long-acting.

Liraglutide (Victoza®, NovoNordisk) was recently investigated as a once daily drug in cats.[174] Over a 14 day study period, liraglutide was shown to significantly increase insulin concentrations during hyperglycemic clamp while also decreasing glucagon concentrations. Liraglutide was also associated with a significant reduction in weight and appetite.

Only one oral DPP-4 inhibitor, sitagliptin (Januvia®, Merck) has been investigated to date in cats. In one report, sitagliptin provided a modest increase in post-prandial serum insulin which is in contradiction to a second report in which no effect of sitagliptin on post-prandial insulin was detected.[172,175] NVP-DPP728 is a small molecule inhibitor of the DPP-4 enzyme that must be administered intravenously. It has been evaluated in one study where it was given to apparently healthy cats.[176] Single dose of NVP-DPP728 was effective at reducing plasma glucagon and increasing plasma insulin in response to a meal. NVP-DPP728 is only available for biochemical research and there are no known plans to develop this compound as a drug for use in the clinical setting.

Our expectations for these drugs to be effective in our feline patients are rooted in the inherent similarities between feline diabetes and Type 2 diabetes in people, in whom incretin-based therapies are commonly used. There are still significant gaps in our knowledge of the

cellular and molecular basis for disease in the cat and it is unknown if incretin therapies will have similar effects on the feline beta cell as have been described in other species.

1.9 Summary and statement of hypothesis

In order to better understand the development of beta cell dysfunction in diabetics and the role of GLP-1 and GLP-1 mimetics in mitigating these changes, we developed the following central hypothesis:

GLP-1 and GLP-1 mimetics have a beta cell sparing effect in feline pancreatic islets by mitigating the dysfunction associated with amyloid, oxidative stress, inflammatory cytokine signaling, and endoplasmic reticulum stress thereby inhibiting cell death.

It is well documented that pancreatic islets from people and rodents have evidence of oxidative stress and inflammatory cytokine signaling associated with exposure to conditions similar to that seen in the developing diabetic, namely hyperglycemia. It is unknown if these same changes are found in the diabetic cat. We hypothesized that ***the pancreatic islets of diabetic cats will have evidence of increased oxidative modification, inflammatory cytokine signaling and amyloid as compared to control cats.*** In order to test this hypothesis we established the following aim:

> *Aim 1*: Characterize the markers of inflammation, oxidative stress, and amyloid in the pancreatic islets of normal and diabetic cats. To achieve this aim, histologic samples from control and diabetic cats were obtained and semi-quantitative measurements of interleukin-1 beta, 4-hydroxynonenal, and amyloid were made.

Efforts to study mechanisms of beta cell dysfunction in the cat are severely hampered by the lack of available techniques to isolate and culture feline islet tissue *in vitro*. We hypothesized that ***feline pancreatic islets can be isolated and maintained in culture without loss of function for a period of two days and with good viability for a period of five days.*** In order to test this hypothesis we established the following aim:

> *Aim 2*: Develop a technique for the isolation and culture of purified feline pancreatic islets suitable for use in functional assays. To achieve this aim, islets from cadaveric cats were isolated using collagenase-based digestion and maintained in culture for a period of 2-5 days. Viability was estimated by fluorescent assay and quantified by LDH release into the media. Islets were subjected to a glucose stimulated insulin production and release assay after two days in culture.

The extremely high demand on beta cell synthesis of proteins (insulin and IAPP) make the pancreatic beta cells particularly susceptible endoplasmic reticulum stress secondary to protein misfolding. We hypothesized ***that exenatide will reduce ER stress-related activation of apoptosis in pancreatic islets exposed to ER stress.*** In order to test this hypothesis, we established the following aim:

> *Aim 3*: Determine the effect of increased ER stress on caspase 3 activity in freshly isolated islets. To accomplish this aim, freshly isolated feline islets were exposed to drugs inducing ER stress and caspase 3 activity was quantified.
>
> *Aim 4*: Evaluate the effects of the GLP-1 mimetic exenatide on caspase activity of freshly isolated islets under conditions of ER stress. To address this aim, doses of drugs identified in Aim 3 were used to induce ER stress and thereby

caspase 3 activity in islets. The treatment effect of co-culture with exenatide and ER stress drug on caspase 3 activity was then quantified.

Finally, the clinical application of the GLP-1 mimetic exenatide was evaluated in newly diagnosed diabetic cats. The benefits of GLP-1 mimetics in human diabetics are well described and the many similarities between feline and human Type 2 diabetes led us to the hypothesis that ***the addition of exenatide to standard insulin therapy will be associated with better glycemic control of newly diagnosed feline diabetics.*** In order to test this hypothesis, we developed the following aims.

Aim 5: Evaluate the effects of adjunctive therapy with exenatide in addition to exogenous insulin in newly diagnosed feline diabetics. To investigate this aim, we recruited newly diagnosed feline diabetics presented to the Boren Veterinary Medical Teaching Hospital. A double-blind, placebo-controlled study design was used and patients were either treated with insulin alone or in combination with exenatide over a 12 week study period.

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CHAPTER 1

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CHAPTER II

OXIDATIVE MODIFICATION, INFLAMMATION, AND AMYLOID IN THE NORMAL AND DIABETIC CAT PANCREAS[1]

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[1] Herndon AM, Breshears MA, McFarlane D. J Comp Pathol. 2014 Nov;151(4):352-62.

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Summary

The pathogenesis of beta cell dysfunction leading to pancreatic beta cell failure seen in type 2 diabetes mellitus is incompletely understood. Pancreatic tissues were collected from nine control cats and nine diabetic cats and stained using immunohistochemical methods for interleukin-1β (IL-1β), insulin, islet amyloid polypeptide (IAPP), and 4-hydroxynonenal (4-HNE). Thioflavin-S was used to stain for amyloid deposits. All control cats showed positive staining for IL-1β and 4-HNE. Diabetic cats showed varying degrees of staining for inflammation and oxidative modification, owing in large part to the very small amount of islet structure remaining in the typical diabetic cat. Amyloid deposition was identified in 8/9 diabetic cats and 1/9 control cats. In order to validate our findings of oxidative modification and inflammatory cytokine signaling in the control pancreases, paired biopsy samples taken (baseline and after 8-16 weeks of obesity and hyperglycemia) from an additional group of cats enrolled in an obesity and hyperglycemia study were stained for IL-1β and 4-HNE. A similar pattern of staining was identified in the baseline samples as seen in control cats. A significant increase in IL-1β and 4-HNE staining was seen after a period of hyperglycemia and obesity. Taken together, these findings suggest that while present in normal cats, markers of inflammation and oxidative modification increase very early on during the development of disease. Future studies focusing on these earlier time-points are needed to understand the factors that function in protection of the islet beta cell and the development of islet pathology in Type 2 diabetes mellitus in the cat.

Introduction

It is estimated that Type 2 diabetes mellitus (T2D) will affect nearly one in three adults in the United States of America by the year 2050 according to one study (Boyle *et al.*, 2010). The domestic feline population has undergone a similarly remarkable increase in morbidity from this

disease. It was estimated that the incidence of T2D in cats was 0.08% in 1970, but by recent accounts is now estimated to be as high as 1.2% (Prahl *et al.*, 2007; Rieder *et al.*, 2008).

The pathogenesis of beta cell dysfunction and failure resulting in T2D is incompletely understood. It is clear that development of disease is not the result of any single mechanism. By the time clinical signs of T2D are manifested, the underlying pathologic processes have been developing for weeks, months, or even years. Diet, genetics, activity, obesity, and environment as well as cellular processes such as accumulation of oxidative damage, inflammation, and misfolded proteins interact, ultimately resulting in a loss of functional beta cell mass and overt hyperglycemia (Boni-Schnetzler *et al.*, 2008; O'Brien, 2002).

Insulin demand is dictated by peripheral insulin sensitivity, dietary glucose intake, and gluconeogenic mechanisms. The early stages of T2D are characterized by relative compensatory hyperinsulinemia in response to peripheral insulin resistance. At some point, the insulin producing capacity of the beta cell is exhausted and a permanent relative or absolute hypoinsulinemic state develops.

Aside from insulin, the other major secretory product of pancreatic beta cells is islet amyloid polypeptide (IAPP), also known as amylin. In health, IAPP is stored in secretory granules at a 1:50 molar ratio to insulin (Westermark *et al.*, 2011). During periods of hyperinsulinemia there is a concurrent rise of circulating IAPP and it has been reported that the serum ratio of IAPP to insulin changes with disease (Gasa *et al.*, 2001; Lutz and Rand, 1996; Mulder *et al.*, 1995; Mulder *et al.*, 1996; Pieber *et al.*, 1993).

In the vast majority of spontaneous T2D in humans and cats, IAPP-derived amyloid is deposited within the islet structures (Johnson *et al.*, 1989; O'Brien *et al.*, 1986). The presence of islet associated amyloid deposits is associated with a loss of beta cell mass and loss of insulin secretion by the pancreas (Höppener *et al.*, 2002; Lutz and Rand, 1997).

Reactive oxygen species are produced as the result of normal aerobic metabolic processes and there is a constant interplay between oxidative and antioxidant forces in the cell. It has been demonstrated that diabetic people have evidence of increased oxidative stress both systemically and locally, and it is believed that oxidative stress is involved in the process of beta cell dysfunction and apoptosis (Acharya and Ghaskadbi, 2010; Bast *et al.*, 2002; Ihara *et al.*, 1999; Li *et al.*, 2009; Modak *et al.*, 2011; Robertson *et al.*, 2004).

Pro-inflammatory cytokine signaling has also been implicated as a central player in the initial maintenance and ultimate destruction of the pancreatic beta cell. It has been proposed that interleukin-1β (IL-1β) signaling is useful in stimulating pro-survival signals through promotion of NF-kB signaling. Through autocrine feedback, prolonged IL-1β signaling eventually pushes the cell in a pro-apoptotic direction and may ultimately be partly responsible for loss of beta cell mass (Boni-Schnetzler *et al.*, 2008). The importance of IL-1β signaling is supported by a clinical study of an IL-1β receptor antagonist that resulted in improved beta cell survival, insulin production, and resolution of hyperglycemia in human subjects (Larsen *et al.*, 2009).

The aim of this study was to characterize markers of oxidative modification, inflammation, and amyloid within the pancreatic islets of normal and diabetic cats. Immunohistochemical and fluorescent staining techniques were employed to detect markers of inflammation (IL-1β, interleukin-6 (IL-6)), markers of oxidative modification (4-hydroxynonenal (4-HNE)), insulin, IAPP, and amyloid. We hypothesize that the islets of diabetic cats will contain evidence of increased oxidative modification, increased inflammation, and amyloid accumulation as compared to islets of normal cats.

Materials and Methods:

Case selection:

Cases of spontaneous diabetes mellitus in cats were identified by reviewing the submission records to the Oklahoma Animal Disease Diagnostic Laboratory in Stillwater, Oklahoma. A diagnosis of diabetes mellitus characterized by spontaneous and persistent hyperglycemia made by the submitting veterinarian and sufficient paraffin embedded tissue available for stains were considered adequate for inclusion into this study. Cases with severe systemic disease such as fungal infection or sepsis were excluded. Additionally, histologic evidence of severe acute or chronic pancreatitis resulted in exclusion of that individual. Nine cases met inclusion criteria for the diabetic group. A summary of signalment, cause of death, and if available, duration of disease is included in Table 1.

A group of apparently healthy research cats (n=4) enrolled in an unrelated study were evaluated before and after induction of obesity and hyperglycemia to serve as an additional control group.

Paired, surgical biopsy samples were taken from individuals at the time of enrollment and again after the cats had been maintained obese and hyperglycemic for a period of time (8-16 weeks).

Pancreases were collected from apparently healthy cats (n=9) killed as a part of routine population control from a local animal shelter. Cats were deemed apparently healthy by shelter staff and showed no signs of respiratory, dermatologic, or neurologic disease during the time they were observed at the shelter. Pregnant individuals were excluded. No exclusions were made based on age or gender. Hematoxylin and eosin stained sections of pancreatic tissue from each case were reviewed by a board certified pathologist. Any evidence of acute or chronic pancreatitis resulted in exclusion from the normal study group.

The samples utilized in this study were from various locations within the pancreas. Previous publications have demonstrated that there is a fairly homogenous distribution of islets within the feline pancreas and so no attempts were made to try to localize the origin of the sample (Lutz *et al.*, 1994a; O'Brien *et al.*, 1993).

Histologic evaluation:

Hematoxylin and eosin stained 4-micron sections of pancreas were examined by a board certified pathologist (MAB) for evidence of acute or chronic pancreatitis or other exclusionary pathology.

Immunohistochemistry:

Sections were re-hydrated by first processing in xylene followed by decreasing ethanol concentrations and finally distilled water. Endogenous peroxide activity was blocked using a 3% hydrogen peroxide bath.

For immunohistochemical staining for IL-1β, 4-HNE, IL-6, IAPP, and insulin, sections were processed using heat induced epitope retrieval. Sections were treated for 15 min at 95°C in a citrate buffered solution (10mM, pH6.0). After 15 min, sections were allowed to gradually cool down for an additional 15 min in the buffer solution before bringing to room temperature in phosphate-buffered saline solution (PBS). Sections were then incubated for 20min in 10% normal blocking serum from the species in which the secondary antibody was raised.

Working concentrations of primary and secondary antibodies were optimized and appropriate negative controls were performed. For negative controls, samples were processed the same as all other samples with the exclusion of the primary antibody. Additional negative controls were conducted for IL-1β, IAPP, and insulin by pre-incubating the primary antibody with the target peptide for 12 hours prior to application of the primary antibody and processing of the sample as described. Primary antibody working concentrations were as follows: anti-feline IAPP antibody (Bachem, Torrance, CA, USA) 1:600, anti-feline insulin antibody (Cell Signaling, Danvers, MA, USA) 1:500, anti-feline IL-1β antibody (R&D Systems, Minneapolis, MN, USA) 2ug/ml, anti-feline IL-6 (R&D Systems, Minneapolis, MN, USA) 6ug/ml, anti-4-HNE antibody (Millipore, Billerica, MA, USA) 1:600.

Samples incubated with anti-IL-1β, IL-6, insulin, and IAPP were incubated with primary antibody overnight at 4°C. Samples for 4-HNE were incubated with primary antibody for one hour at room temperature. Secondary antibodies directed to the primary antibody (Vector Laboratories, Burlingame, CA, USA) were incubated for one hour at room temperature. A streptavidin amplification step (Vectastain ABC, Vector Laboratories, Burlingame, CA, USA) was utilized and chromogens (Immpress Novared and DAB, Vector laboratories, Burlingame, CA, USA) were used as indicators.

Digital images were scanned using a digital microscope system (Aperio ScanScope CS2, Leica Biosystems, Nussloch, Germany). Snapshots of higher magnification images were captured using image software (Aperio ImageScope, Leica Biosystems, Nussloch, Germany). For calculation on insulin and IAPP staining, the entire area of pancreas visible on the section was captured as 10x snapshots (average 10 images) were then used in image processing and quantification. For calculation of 4-HNE and IL-1β, 40x snapshots of between 10 and 20 morphologically identifiable islets or islet remnants were captured and then used in image processing and quantification.

Thioflavin-S staining

After re-hydration as described above, sections were incubated for one minute at room temperature in a solution of 1.0% thioflavin-S (Sigma Aldrich, Saint Louis, MO, USA) in PBS. Sections were rinsed briefly three times in 70% ethanol and then in distilled water. Coverslips were mounted using glycerin jelly. Sections were examined using a Nikon Eclipse 50i

microscope at an excitation wavelength of 450-490nm, and an emission wavelength of 495nm. Images were taken using a Nikon Digital Sight DS-L1 camera.

Image processing and staining quantification:

All images were processed using a software program (ImageJ, NIH, Bethesda, MD, USA) and quantified by one observer (AMH) who was masked to sample identity. Quantification of IAPP, insulin, and thioflavin-S, was accomplished using the software program and the following procedure: Briefly, all non-parenchymal areas of the image were trimmed away and the area of pancreas was calculated using calibrated measurements. Images were then converted to a 32-bit grayscale image and the "threshold" function was used to filter out areas of non-staining or insufficient staining intensity. The results are reported as a ratio of positive staining *per total area of pancreas* measured.

To quantify intensity of staining of 4-HNE and IL-1β the surface area of individual islets captured was calculated and the same techniques of grayscale conversion and image thresholding were used to calculate the proportion staining positive for 4-HNE or IL-1β. The results are reported as a ratio of positive staining *per islet* in the sample.

Statistical Analysis

Differences between groups were evaluated using a software program (GraphPad Prism, GraphPad Software, Inc, LaJolla, CA, USA). Non-normally distributed data was transformed using negative natural log. Log-transformed and normally distributed variables (insulin, IAPP,

IL-1β, 4-HNE) were compared using a Student's t-test. Non-normally distributed data (thioflavin-T) was compared using a Mann-Whitney U test. Differences were considered significant when the calculated p-value was <0.05.

Results:

Hematoxylin and eosin stain:

A representative image of an islet from the control group is seen in Figure 2.1A. Histopathologic changes were found in only one control cat and included deposition of amorphous, eosinophilic material effacing occasional islets (amyloid).

Common histopathologic findings in the H&E stained pancreas sections in the diabetic group included nodular exocrine pancreatic hyperplasia, vacuolated islets, lack of morphologically identifiable islets, and amorphous, eosinophilic material effacing islets (amyloid) (Fig. 2.1B).

Insulin

Representative images of pancreas stained for insulin are seen in Figures 2.2A-B. Insulin staining was significantly reduced in the diabetic group compared to control ($p<0.0001$), with a mean ratio of 0.0253 (range 0.00083-0.0843 insulin positive/total pancreas area) in the diabetic cats and 0.4195 (range 0.2813-0.5425 insulin positive/total pancreas area) in the control cats (Fig. 2.2C). Cells staining positive for insulin were nearly all associated with a morphologically identifiable islet in the normal group and in most of the diabetic group (Fig. 2.2A). In the diabetic group it

was also common to see individual or small clusters of cells staining positive for insulin outside of any identifiable islet (Fig. 2B).

IAPP

Representative images stained for IAPP are seen in Figures 2.3A-B. The antibody for IAPP recognized both unfolded, native IAPP within the beta cell and the fibrillar amyloid deposited in the tissues around the beta cell. Therefore, the proportion of pancreas staining positive for IAPP varied greatly depending on the presence or absence of amyloid within the islet, with a mean IAPP/pancreas area of 0.0558 (range 0.00115-0.2596) in diabetic cats and 0.01367 (range 0.0106-0.0207) in the control cats ($p=0.53$). In the normal cats, IAPP staining was localized to the islet beta cells. When amyloid was present, the IAPP antibody produced a diffuse darkening of the amyloid deposits but never with the intensity associated with the staining of a beta cell containing native IAPP.

Thioflavin-S

Deposits of amyloid could be easily identified (Fig. 2.4A-B). The presence of mild background fluorescence did not affect the detection of positive signal or affect calculations using the image software. The mean proportion of pancreatic area staining positive with thioflavin-S was greater in diabetic cats ($p=0.001$) with a mean ratio of 0.03111 (range 0.0 to 0.075) in diabetic cats and 0.000464 (range 0.0 to 0.0042) in control cats.

IL-1β

Representative images stained for IL-1β are seen in Figures 2.5A-B. Positive staining for IL-1β was found in nearly all islets. In normal islets, the IL-1β staining was limited to cells around the periphery of the islet consistent with beta cells (Fig. 2.5A). In diabetic cats, IL-1β staining was found in cells associated with vacuolated islet structures (Fig. 2.5B). We were not able to confirm the identity of these cells (mononuclear cell or beta cell). There was significantly less staining for IL-1β in diabetic cats with a mean proportion of positive staining per islet area of 0.0754 (range 0.0097-0.311) in diabetic cats and 0.35 (range 0.174-0.573) in control cats ($p<0.001$. Fig. 5F).

IL-6

In order to confirm the presence of inflammatory cytokine signaling within the islet, sections of pancreas from a subset of cats in each group were randomly selected and stained for IL-6. Similar to IL-1 β, IL-6 staining was found in nearly all islets. In normal islets, IL-6 was localized in anatomically identical islet location presumed to be beta cells (Fig. 2.5H).

4-Hydroxynonenal

Representative images stained for 4-HNE are presented in Figures 2.6A-E and 2.6G. 4-HNE staining was found in islets from nearly all cats. There was significantly less staining for 4-HNE in diabetic cats with a mean proportion of islet staining positive for 4-HNE of 0.236 (range 0.04-0.67) in diabetic cats and 0.4127 (range 0.235-0.555) in control cats ($p=0.05$). Staining for 4-HNE was strongest in the islet periphery in the region of the beta cells and in association with amyloid deposits when present.

Additional control group

The finding of large amounts of inflammatory cytokines and oxidative modification in the islets of normal cats was unanticipated. Since these control cats were all sourced from a shelter and had unknown extended medical histories it is possible the control cats were not truly normal. Furthermore all samples in this study were collected from individual animals at postmortem examination resulting in marked inter-animal variation. Tissues from the cats prior to induction of obesity and hyperglycemia (baseline) also showed extensive positive staining for inflammatory cytokines and oxidative modification (Figs 2.5C-D, and 2.6D-E). Paired samples from the study endpoint (post) showed a significant increase in both IL-1 β ($p=0.004$) and 4-HNE ($p=0.006$) staining in islets from the same individual after a period of obesity and hyperglycemia (Figs. 2.5G, 2.6F).

To explore the possibility that oxidative modification and inflammatory cytokine staining was a phenomenon of older cats, the pancreas of a kitten approximately 4 months of age was stained for IL-1β and 4-HNE. These sections demonstrate a similar, although less intense, pattern of positive staining (Figs. 2.5E and 2.6G).

Discussion:

The aim of this study was to characterize markers of oxidative modification, inflammation, and amyloid within the pancreatic islets of normal and diabetic cats in order to identify potential mechanisms involved in the development of pancreatic beta cell dysfunction.

One of the interesting findings from this study was the evidence of both oxidative modification and inflammatory cytokines in the islets of normal individuals. Oxidative modification is associated with an imbalance in the production of reactive oxygen species (ROS) such as nitric oxide, hydroxide ion, peroxide, and superoxide, relative to the anti-oxidant capacity of the cell. ROS are normally produced as a by-product of many different cell functions. However, in states of increased inflammation or metabolic derangements, the production of ROS can exceed the anti-oxidant capacity of the cell. The resulting excess free radicals produced can react with a variety of lipids and amino-acids. For example, lipid peroxidation results in formation of oxidized lipid compounds such as 4-hydroxynonenal. In this study, the marker of lipid oxidative modification, 4-HNE, was selected because of the good performance of antibodies directed towards these epitopes.

In people, the pancreas is considered one of the least well-endowed organs in regards to overall antioxidant status (Robertson *et al.*, 2004). The results of our current study suggest that the beta cells may be particularly sensitive to oxidative modification since extensive oxidative modification was found in many of the normal cats. Oxidative stress is one proposed mechanism of beta cell dysfunction and has been indicated as a trigger of apoptosis(Donath *et al.*, 2005). Generation of ROS and imbalance of the REDOX potential of the cell is also implicated as a potential second messenger system for the beta cell. The beta cell's metabolic rate is dependent on the influx of glucose from the surrounding environment via the constitutively active GLUT-2 transporter. Excessive energy available to the cell will result in additional ROS generation by the mitochondria via oxidative phosphorylation. The increase in ROS has been associated with changes in gene expression, in particular up regulation of uncoupling protein-2 which is believed to be protective to the beta cell by uncoupling oxidative phosphorylation and reducing oxygen radical generation. The finding of oxidative modification in beta cells of the control cats and in

the kitten suggests that oxidative modification may be a characteristic of the normal beta cell. Furthermore, the increases in oxidative modification and oxidative stress seen in hyperglycemic and obese cats may indicate that these are early events in the pathogenesis of beta cell dysfunction in the cat.

IAPP is a 37-residue polypeptide hormone produced by the pancreatic beta cell and co-processed, packaged, and released with insulin in response to increased serum glucose concentrations. Like humans and non-human primates, feline IAPP is capable of misfolding and producing large, extracellular conglomerations of fibrillar amyloid in the tissues surrounding the pancreatic islet. The precise mechanisms of IAPP mis-folding are incompletely understood, although the peptide sequence from amino acid positions 25-29 appears to be critical for the production of amyloid. In cats and people, this sequence allows folding of the peptide into beta-pleated sheets under appropriate conditions. Native murine IAPP is unable to spontaneously fold and it is believed to be due to substitution of multiple proline residues within positions 25-29 of the peptide (Westermark *et al.*, 1990). Inappropriate peptide processing as well as vesicular pH, zinc concentration, and molar concentration ratio of IAPP to insulin have all been proposed as possible mechanisms promoting misfolding of IAPP into multimeric amyloid (Brender *et al.*, 2010; Jaikaran and Clark, 2001).

Once produced, lower-order multimers of amyloid (tetramers, decamers, etc) are suspected to be directly toxic to cells largely via disruption of cell membrane integrity (Meier *et al.*, 2006; Weise *et al.*, 2010; Zhao *et al.*, 2009). As the misfolded proteins continue to accumulate, they fold into larger, fibrillar amyloid which is deposited into the surrounding tissues. It is this fibrillar amyloid

that is visible with standard histopathology as amorphous material effacing normal islet architecture.

In our study, amyloid deposits were found in 8/9 diabetic cats and in 1/10 control cats. These findings are consistent with previous reports that suggest amyloid deposition is common in, but not a pre-requisite for development of T2D in the cat (Ma *et al.*, 1998). These findings are inconsistent with reports describing 83 random-sourced cats in Queensland, Australia, in which greater than 90% of apparently healthy, random-sourced cats had amyloid deposits within their pancreas (Lutz *et al.*, 1994b). It is impossible to draw conclusions about the population based on this small sample set, but one could postulate that differences in amounts of amyloid found in the pancreas of normal cats may be heavily influenced by regional population genetics and diet.

Local paracrine and autocrine inflammation is also considered a key component in the loss of beta cell mass. In this study, we confirmed the accumulation of pro-inflammatory cytokines IL-1β and IL-6 in the pancreatic islet, presumably within the beta cell. Increased staining for IL-1β in obese, hyperglycemic cats suggests that pro-inflammatory cytokine signaling may be involved in the mechanism of pancreatic beta cell dysfunction. The possible importance of IL-1β signaling in beta cell pathogenesis is supported by studies in people using a novel IL-1 β receptor antagonist that have shown a return to normoglycemia in diabetic patients taking the drug (Larsen *et al.*, 2009).

Positive staining for insulin was found in all samples including the diabetic animals. Type 2 diabetic cats experience a combined peripheral insulin resistance as well as gradual decline in

pancreatic insulin production capacity, so it is not surprising that there is a very small residual insulin producing capacity in even long-standing diabetic cats. The vast majority of the insulin staining in the pancreas of the diabetic cats was not within an identifiable islet structure. Instead these individual or small clusters of cells were seen scattered in the parenchyma. These "orphaned" beta cells may have been derived from islet-associated beta cell replication or may be the result of beta cell neogenesis from pancreatic ductal epithelium (Hui *et al.*, 2001; Xu *et al.*, 2008).

The samples selected for the normal control group originated from a group of shelter cats being euthanized during routine population control. Although each individual was identified as apparently healthy, it was not possible to complete a comprehensive physical examination or biochemical analysis to rule out systemic illnesses. As such, it is possible that cats in the normal group may have had some degree of subclinical illness that was undetected. Precise measurements of age were not possible as each control cat was identified as a "stray." Based on dental examination and overall body size and condition, it was estimated that all cats were young adults, probably less than five years of age. It is possible that one or more of the cats may have been older than they appeared.

Age and recent medical history were known for each of the cats in the diabetic group. Six cats in the diabetic group died as a direct result of complications from diabetes. One cat died of respiratory failure due to pulmonary neoplasia, another cat died of congestive heart failure, and a third died of mast cell neoplasia. The presence of comorbidities could theoretically impact the finding of oxidative modification or inflammation. Two of the diabetic cats were newly diagnosed and died shortly after diagnosis as the result of complications of their disease (diabetic

ketoacidosis for one and hyperglycemic-hyperosmolar syndrome in the other). Another of the diabetic cats had only periodically required insulin therapy over the year prior to death. At the time of death the patient was receiving parenteral insulin therapy. The variability in this population may explain some of the variability in our findings. A similar study in a larger group of cats may help better describe if certain pathologic changes are more specific to a particular subset of cats.

The pancreata studied from diabetic cats likely represents an "end stage" of disease. Pancreatic islets were typically missing, heavily vacuolated, or completely effaced by amyloid in the diabetic cats. There was profound loss of beta cell mass and so identifying the role of oxidative modification or inflammation during the course of disease progression was impossible. Study of a large number of clinically normal cats who are at high risk of T2D such as morbidly obese cats may provide an opportunity to "catch" disease as it progresses and better help describe the mechanisms active during the gradual development of T2D in a way that this study cannot. It was for this reason that the group of study cats was used as a comparison. The hope was that the better-known and strictly controlled health status of the study cats would help eliminate some of the variability inherent to cats sourced from the shelter. Despite the differences in their history, both groups of cats had clear presence of oxidative modification and inflammatory cytokines within the islets. Even more interesting was the finding that the study cat group saw an overall increase in these markers in the islet after they were obese and hyperglycemic for several months.

Immunohistochemistry is, at best, semi-quantitative and this is a major disadvantage of this study. It is impossible to measure actual quantity of protein or evaluate function. Detection by antibody of IL-1, IL- 6, IAPP, and insulin does not allow any definitive statements regarding biological

functionality of these hormones and cytokines. The antibodies used could detect immature hormone or cytokine and therefore cannot discriminate between active or inactive peptides. In this study, IHC was used because it provided a very useful semi-quantitative technique for retrospective study where protein quantification, function, or gene expression experiments were impossible to perform. Additionally, IHC allows localization of staining and morphometric identification of islets versus exocrine tissues.

In the current study, we have shown that oxidative modification, inflammatory cytokine signaling, and misfolded proteins accumulate in the beta cells of both normal and diabetic cats. These findings, together with our findings from a subset of apparently healthy study cats, suggest that inflammation and oxidative modification are present in normal cats and that their expression increases very early on during the development of disease. Future studies focusing on these earlier time-points are needed to understand the factors that function in protection of the islet beta cell and the development of islet pathology in Type 2 diabetes mellitus in the cat.

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Conflict of Interest:

The authors have no conflicts of interest to declare.

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Table 1: Summary of available data for the nine diabetic cats

Cat number	Sex	Age	Breed	Time Since Diagnosis of T2D	Cause of death
1308	MN	Unknown	DSH	6 weeks	Diabetic ketoacidosis
0913	MN	12yr	DSH	Unknown. On insulin therapy	Hypertrophic cardiomyopathy
1270	FS	7yr	DSH	4 days	Diabetic ketoacidosis
0990	MN	16yr	DSH	10 weeks	Hyperthyroidism. Hypernatremia presumably secondary to functional adrenal neoplasm
0723	MN	14yr	SDH	3 days	Hepatic lipidosis. Humane euthanasia
1174	SF	10yr	DSH	>1year	Mastocytosis
1354	SF	13yr	DSH	Unknown – on insulin therapy	Undetermined. Found dead.
0149	MC	13yr	DLH	>6mo	Poorly regulated on insulin therapy. Humane euthanasia
1694	MC	12yr	DSH	1 day	Owner elected humane euthanasia in lieu of treatment.

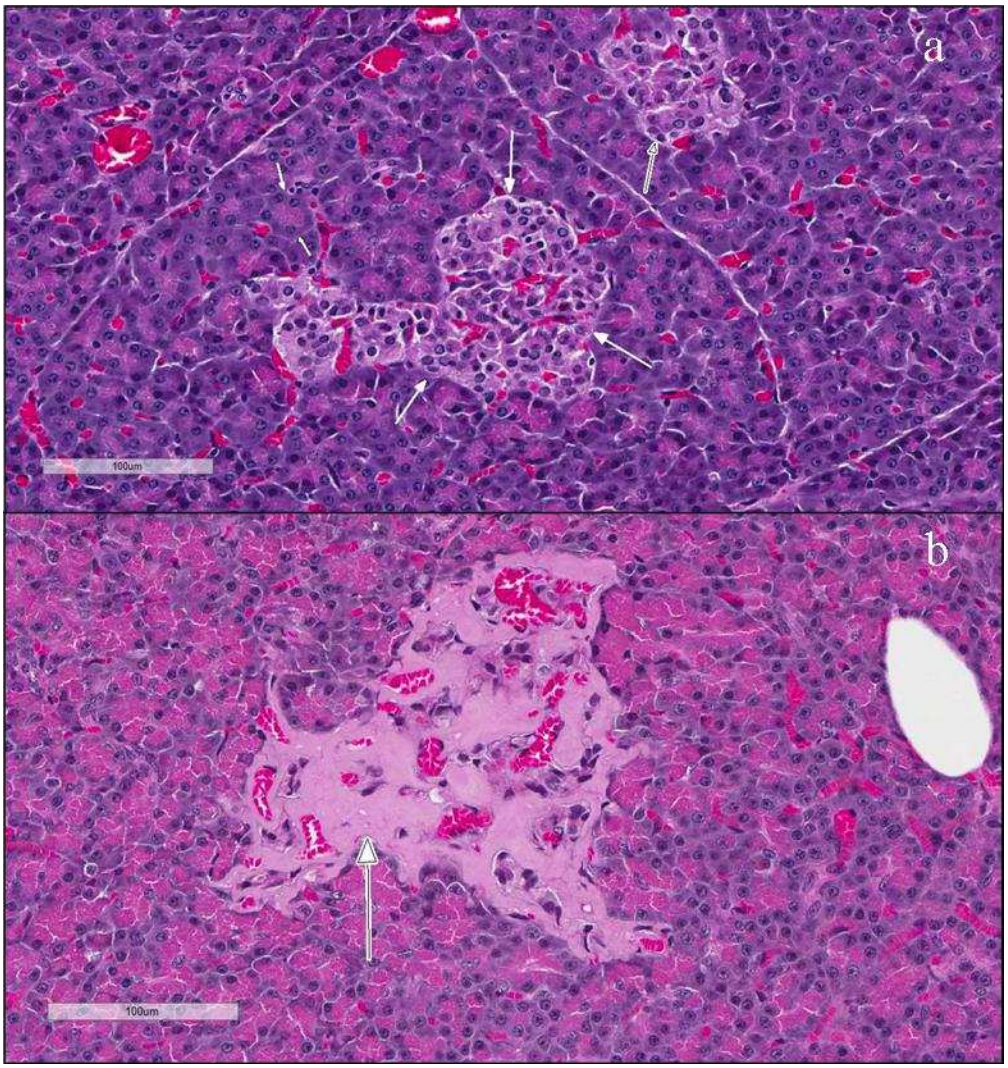

Figure 2.1. Representative section from a control cat (A) and diabetic cat (B). The control islet has normal morphology with a typical islet structure indicated by the large arrows. The endocrine islet is surrounded by the exocrine acini (small arrows). In the diabetic cat the islet has been completely effaced by the amorphous material (amyloid) noted by the arrows. HE. Bar, 100µm

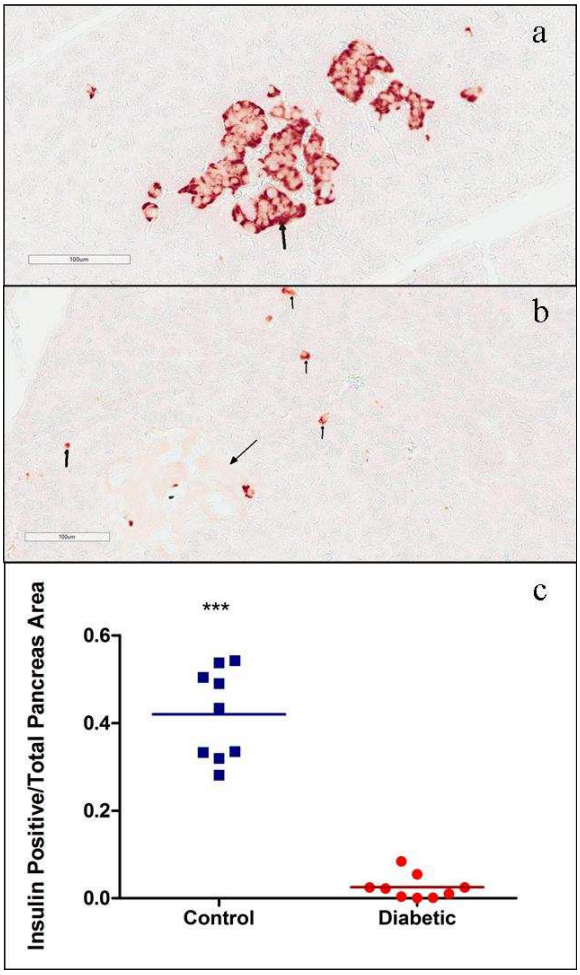

Figure 2.2. Insulin staining of pancreas from a control cat (A) shows the insulin positive cells to be largely around the periphery of the islet. In the diabetic cat (B), individual and clusters of insulin positive cells are seen within the adjacent parenchyma (small arrow) and only a few insulin positive cells remain within an islet that is effaced by amyloid (large arrow). Fig. 2C compares the mean insulin positive area per total pancreas area between control and diabetic cats ($p<0.0001$). IHC. Bar, 100µm

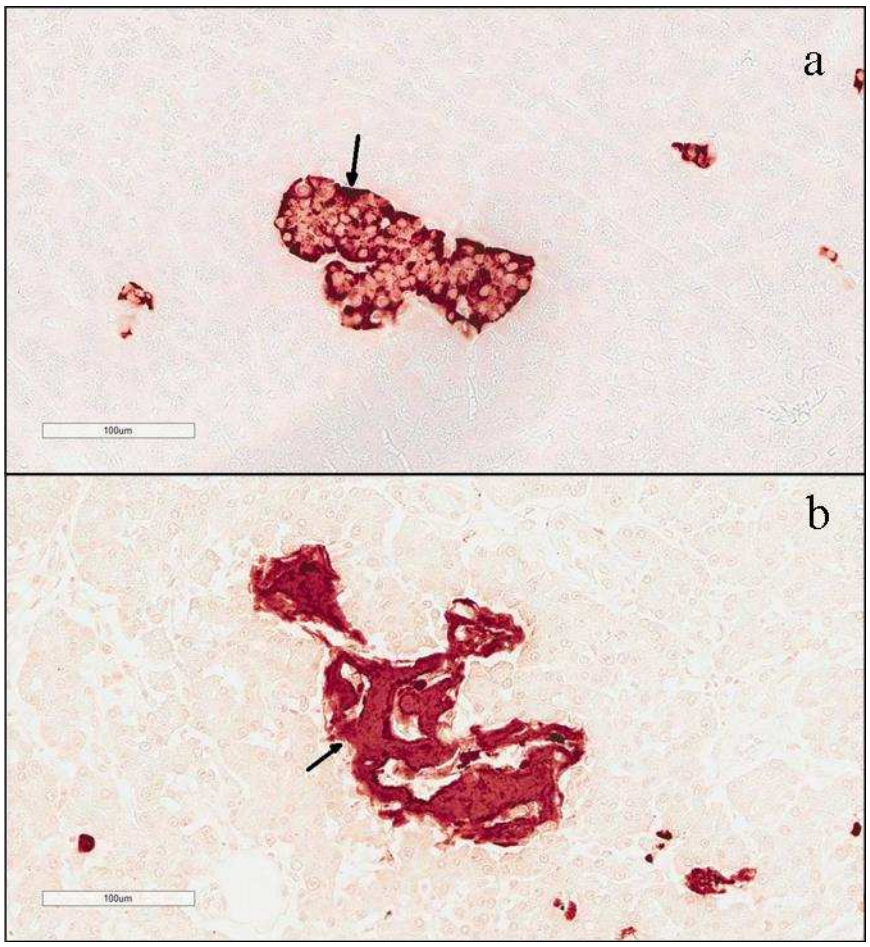

Figure 2.3. IAPP staining of the pancreas from a control cat (A) shows the IAPP positive cells to be largely around the periphery in a pattern identical to that seen with insulin (arrow). In the diabetic cat (B), however, the deposits of amyloid stain less intensely for IAPP (arrow) while a few IAPP positive cells are scattered within the parenchyma. IHC. Bar, 100µm

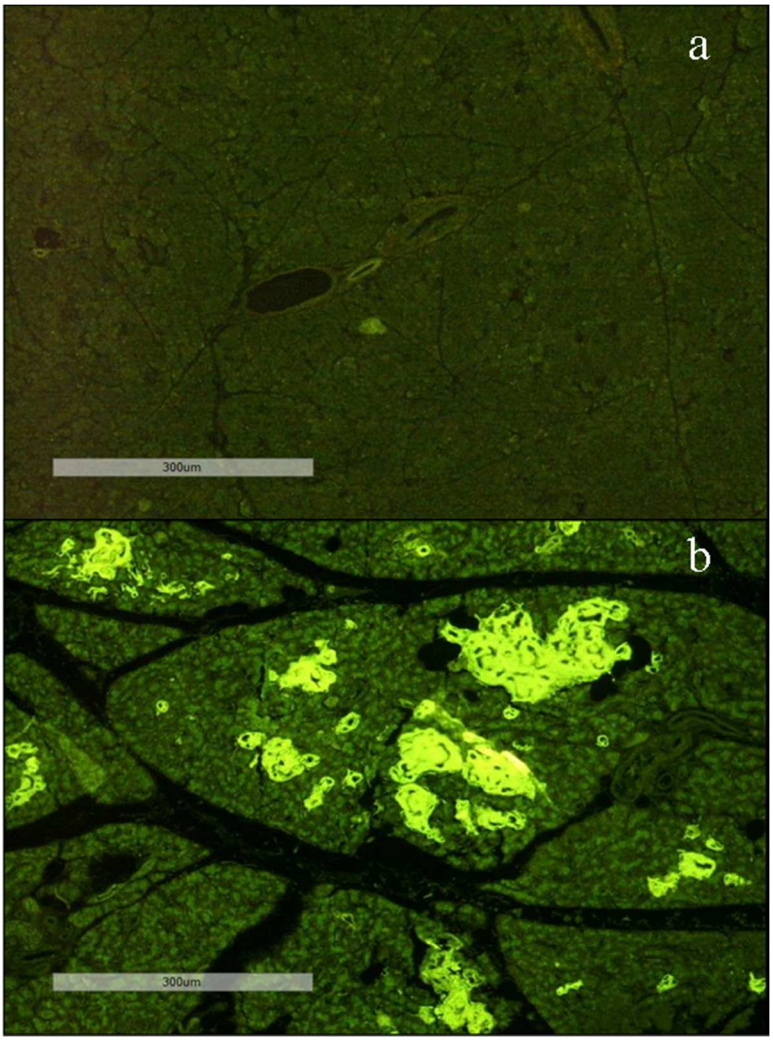

Figure 2.4. Thioflavin-S stained sections of a pancreas were used to identify deposits of amyloid. A typical image of a pancreas from a control cat with no amyloid (A) in comparison to a pancreas from a diabetic cat with extensive amyloid deposits (B). Bar, 300µm

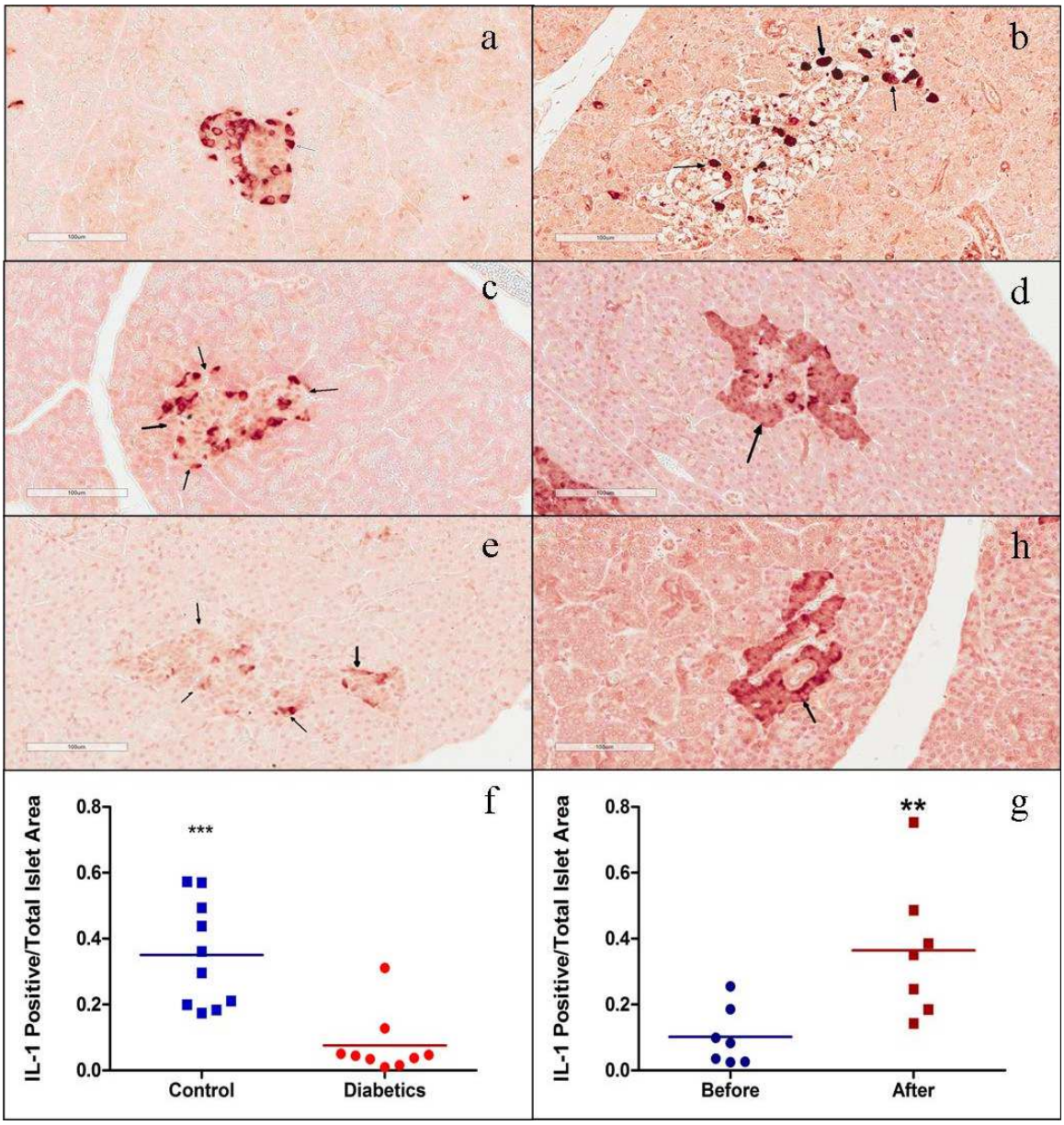

Figure 2.5. Staining for IL-1β varied greatly between groups. In control cats (A) the staining is around the periphery and consistent with beta cells (arrow). In diabetic cats (B) there is rare IL-1β staining in islet remnants. In the additional control cats, there is a clear difference in the staining intensity from baseline biopsy (C) and post biopsy taken after sustained obesity and hyperglycemia (D). Islets of a four month old kitten also show positive IL-1β staining in islets, although with less intensity as was found in control cats (E). Fig. 5F compares mean area

staining positive per total islet area between control and diabetic cats ($p<0.01$). Fig. 5G compares mean area staining positive IL-1β per total islet area between samples taken at baseline and post treatment in the additional control group ($p=0.004$). To support the presence of inflammatory cytokines in the control islets, a subset of sections from control cats were stained for IL-6 (H). Cells around the periphery of the islet stain positive for IL-6 in a similar pattern to that seen for IL-1β. IHC. Bar, 100μm

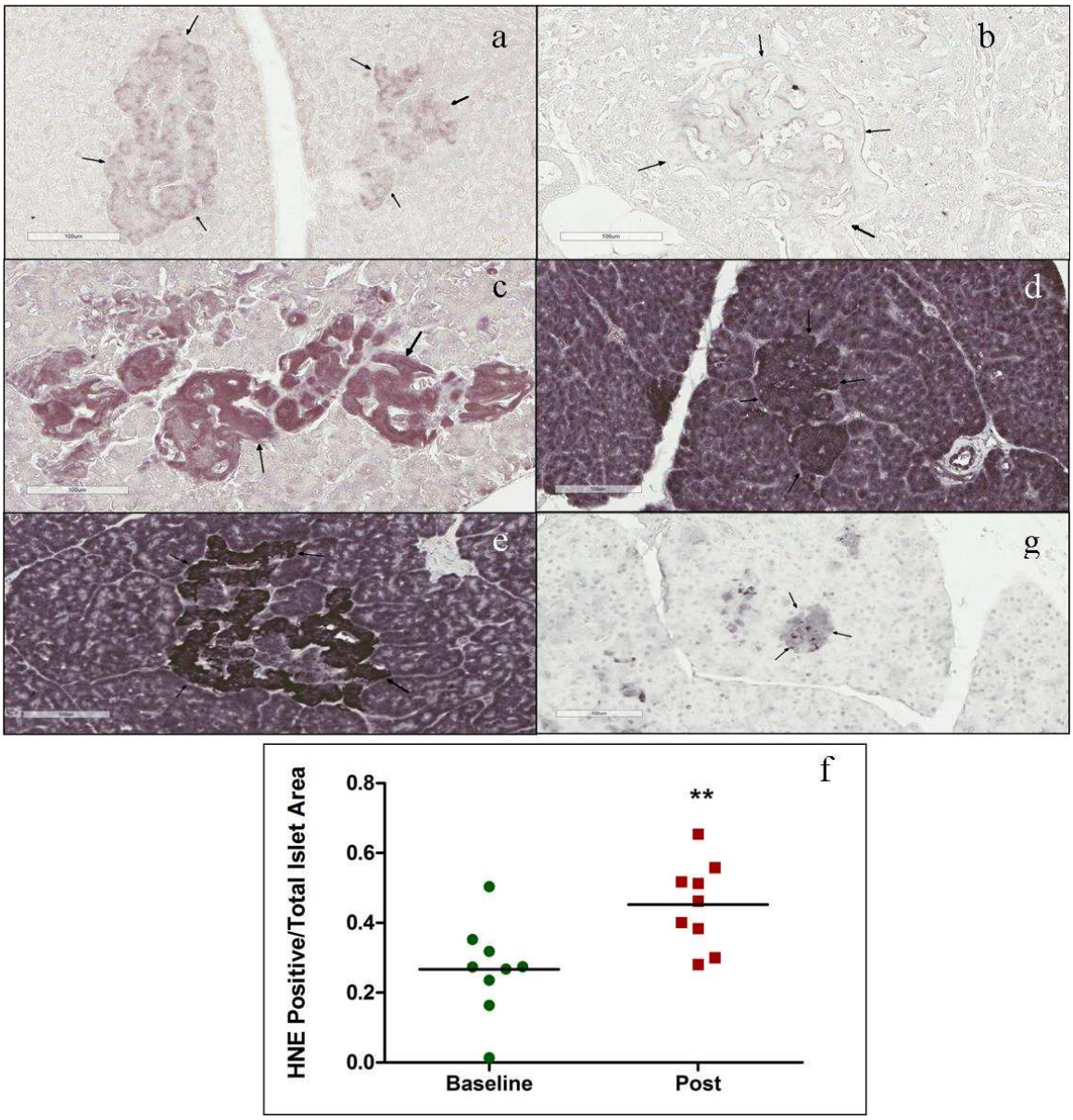

Figure 2.6. Oxidative modification was identified in nearly all islets from normal cats (A) and is highlighted by the arrows. In diabetic cats there was a more varied appearance of oxidative modification. In some islet remnants with amyloid there was no positive staining (B), while in others there was extensive staining for 4-HNE (C). In the additional control cats, there is a clear difference in the degree and intensity of staining found in the samples from baseline (D) and after sustained obesity and hyperglycemia (E). Fig. 6F compares mean area staining 4-HNE positive

per total islet area between samples taken at baseline and post treatment in the additional control group ($p=0.006$). Evidence of oxidative modification was also seen in a young kitten (G), suggesting that oxidative modification may be a normal finding, even early in life. IHC. Bar, 100μm

CHAPTER III

ISOLATION AND CULTURE OF FELINE PANCREATIC ISLETS

3.1 Introduction

Efforts to better understand the mechanisms underlying beta cell dysfunction accompanying the development of feline diabetes mellitus have been complicated by a lack of species-specific cell lines and tissue culture protocols. Although insulin synthesis, demand, and sensitivity of peripheral tissues are influenced by a number of neurohormonal factors, the beta cells within the pancreatic islets of Langerhans are solely responsible for the production of insulin and beta cell loss of function is a prerequisite to development of clinical disease. Therefore, the ability to study isolated pancreatic islet cells *in vitro* is important in unraveling the pathophysiology of feline diabetes.

Islets of Langerhans are small, discreet islands of endocrine cells distributed throughout the pancreas. Islets are composed of five primary cell types, each with different primary secretory products: alpha cells produce glucagon, beta cells produce insulin and islet amyloid polypeptide, delta cells produce somatostatin, epsilon cells produce ghrelin, and PP cells produce pancreatic polypeptide. There is considerable paracrine interaction between the cells of the pancreatic islet, most notably between alpha and beta cells. Because of the relative complexity of the islet microenvironment and the likelihood for paracrine interaction among component cell types, it is valuable to study beta cells while still maintaining islet integrity.

Isolated pancreatic islets from people, rodents, and pigs are commonly used in research and procedures for isolation and culture of these islets are well described in the literature.[1-6] Most procedures require a collagenase digestion of the organ and either density centrifugation or manual selection of individual islets to separate islet material away from exocrine tissue. Yields in the hundreds or thousands of islets per pancreas are possible in some species.[7,8] Attempts to isolate pancreatic islets from cats have been less successful than those described in other species, with poor yields of islets clean of exocrine tissue.

The purpose of this study was to describe a technique for the isolation and culture of feline pancreatic islets. We hypothesize that feline pancreatic islets can be isolated and maintained in culture without loss of function for a period of two days and with good viability for a period of five days.

3.2 Materials and methods

Fresh pancreases were collected from random-source cats euthanized at a local animal shelter as part of a routine population control program. Cats of any age greater than six months were included. The cats were identified as apparently healthy at the time of euthanasia by shelter personnel. Individuals diagnosed as pregnant or lactating and those with severe systemic disease or injury (e.g. excessively thin, life-threatening injury, severe gastrointestinal or respiratory illness) were excluded. Euthanasia was performed by shelter staff using a pre-medication of xylazine (AnaSed, 16.6mg xylazine/cat, Lloyd Labs, Shenandoah, IA, USA) and ketamine (Ketalar, 83mg ketamine/cat, Henry Schein® Animal Health, Dublin, OH, USA) followed by intravenous overdose of barbiturate medication (Fatal-Plus, 1170mg pentobarbital/cat; Vortech Pharmaceutical, Ltd, Deerborn, MI, USA). Islet isolation and stimulation was completed in 10 cats including one neutered male, four intact males, two spayed females and three intact females. All cats were estimated to be adults between the ages of two and eight years.

3.2.1 Organ removal

Pancreases were collected from cadaveric cats immediately after euthanasia. The cranial mesenteric vein was isolated and catheterized with a 24 gauge polypropylene intravenous catheter. Forty to sixty milliliters of ice-cold organ storage solution (Belzer UW® Cold Storage Solution, Bridge to Life, Columbia, SC, USA) was slowly infused. The pancreas was then gently removed using a combination of blunt and sharp dissection. The pancreas was transported while bathed in cold storage solution on ice. Cold ischemic time elapsed from organ collection to initiation of collagenase digestion was between 90 and 240 minutes.

3.2.2 Isolation of purified islets and initial culture

Dithizone solution was prepared by mixing 33mg dithizone (Sigma-Aldrich, St. Louis, MO, USA) in one milliliter absolute ethanol (Sigma-Aldrich, St. Louis, MO, USA) followed by alkalization using three drops 10N sodium hydroxide (Sigma-Aldrich, St. Louis, MO, USA). After thoroughly vortexing, the solution was added to 300ml PBS and pH was slowly adjusted to a final pH of 7.4 using hydrochloric acid (Sigma-Aldrich, St. Louis, MO, USA).

Collagenase solution (Liberase TL Research Grade, Roche Life Science, Indianapolis, IN, USA) was prepared in 48ml warmed (37°C) Hanks' Balanced Salt Solution (HBSS; Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 1.081 Wünsch units/ml. The prepared collagenase was injected into the pancreas via trans-capsular injection in multiple sites. Approximately 30ml prepared solution was required to inflate the pancreas. The pancreas was then incubated with the remaining solution in a 37°C orbital shaker water bath at 300rpm. The pancreas was first inspected after 20min and digestion was continued until the pancreas visually appeared adequately digested (typically 25min).

The entire digested pancreas was passed through a 0.5mm wire mesh strainer and rinsed with four volumes ice-cold HBSS with 10% fetal calf serum (Gibco, LifeTechnologies, Grand Island, NY, USA). The filtered material was allowed to rest 5min on ice and then all visible material accumulated on the bottom was transferred to a pair of 50ml conical vials and centrifuged at 300xg for 5min. The supernatant was discarded while leaving the lower 5ml untouched (to avoid disturbing the cell pellet) and the pellet was re-suspended in 40ml ice-cold HBSS with 10% FCS and centrifuged again.

The upper one half of the washed pellet was suspended in 15ml prepared dithizone and 15ml room temperature HBSS with 10% FCS and incubated for 10min. Individual islets free of exocrine pancreatic tissue were then handpicked using an inverted microscope and a micropipetter. Groups of 50 islets were embedded in 75µl extracellular matrix material (Matrigel®, Corning®, Tewksbury, MA, USA) and plated into 24-well tissue culture treated polystyrene cell culture plates (Costar®, Corning®, Tweksbury, MA, USA). Islets were allowed to recover 24h in media consisting of CMRL-1066 (Gibco, LifeTechnologies, Grand Island, NY, USA) containing 34ng/ml IGF-2 (R&D Systems, Minneapolis, MN, USA), 6.25ng/ml selenium (Sigma-Aldrich, St. Louis, MO, USA), 6.2µg/ml transferrin (Sigma-Aldrich, St. Louis, MO, USA), 100units/ml penicillin (Gibco, LifeTechnologies, Grand Island, NY, USA), 100µg/ml streptomycin (Gibco, LifeTechnologies, Grand Island, NY, USA), and 10% fetal calf serum (Gibco, LifeTechnologies, Grand Island, NY, USA). For functional assays, media was exchanged after the first 24h for stimulation media. For viability studies, media was changed at 24h and then every 48h with either a serum-containing or serum-free CMRL-1066 media as described above.

3.2.3 Islet viability and functionality

Viability of islets was assessed using qualitative examination of intact islets after staining with a commercially available calcein am/ethidium bromide assay (LIVE/DEAD®, Life Technologies, Grand Island, NY, USA). LIVE/DEAD® viability was assessed at five days post isolation in islets grown in both serum-containing and serum-free media.

Release of lactate dehydrogenase (LDH) into the media as the result of cell death was also used as a measure of islet viability. Media was changed after a 24h recovery period and replaced with serum-free CMRL-1066. Media was changed on day three and five and the proportion of total LDH measured in the media was compared to that remaining in the cell pellet using a commercially available LDH assay (Cytotox 96 ® Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI, USA). Percent viability was expressed as $[LDH_{cell\ pellet}/ (LDH_{cell\ pellet} + LDH_{media})] * 100$.

Islet functionality was evaluated 24h after isolation using glucose stimulated insulin production and release. After recovery media was removed, the cells were incubated 15min in freshly prepared Krebs-Ringer Bicarbonate Buffer (115mM NaCl, 4.7mM KCl, 1.28mM CaCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 1.0µMol HEPE, 24mMol $NaHCO_3$, pH 7.4) with 1.4mMol glucose. Following this wash step, islets were incubated 120min in KRBB 1.4mMol glucose (pre-stimulation). After two hours, the media was exchanged and islets were incubated in either KRBB with 20mMol glucose (stimulation buffer) for 24 hours or fresh KRBB with 1.4mMol glucose (basal buffer) for 24 hours. Insulin content of the media and of the cell pellet was determined using a commercially available feline insulin ELISA (Mercodia, Uppsala, Sweden) and was normalized to the total protein content of the pellet (µg/ml). Insulin release into the media after stimulation was available for eight of the cats whereas insulin content of the cell pellet was available for all ten cats.

3.2.4 Statistical Analysis

All non-parametric data was normalized by natural log transformation. For islet stimulation assays, a two-tailed, paired, student's t-test was used to test for significance between the mean stimulated and resting insulin content of the cell pellet. Differences between groups were evaluated using a software program (SigmaPlot, Systat Software Inc, San Jose, CA, USA). Differences were considered significant when the calculated p-value was <0.05.

3.3 Results

3.3.1 Islet yield and purity

A typical pancreas yielded 150 islets completely free of exocrine tissue, although actual numbers varied between 125 and 300. (Figure 3.1a)

3.3.2 Islet viability

Islet viability after five days maintained in serum-containing media was estimated to be greater than 90% based on visualization of intact islets stained with LIVE/DEAD®. (Figure 3.2a-b) In contrast, islets cultured in serum-free media had larger numbers of ethidium staining nuclei within the islets, indicating more cell death. (Figure 3.2c-d) Islets that survived the first 24 hours after recovery tended to remain intact and morphologically unchanged over the five days of culture. When viability of islets cultured five days in serum-free media was estimated based on release of LDH, islet total cell viability was calculated to be 77%.

3.3.3 Glucose stimulated insulin release

Glucose stimulated insulin release into the media was completed for eight cats (n=3 intact males, n=5 intact female) and a wide range of responses were produced. Some cats showed a robust response and others showed no effect (or a negative effect) of glucose stimulation. The difference of magnitude change in media between stimulated and unstimulated controls was not significant. ($p=0.67$, Figure 3.3) To investigate whether the lack of consistent responses could be

related to a failure of insulin trafficking and release from the beta cell rather than failure of transcription/translation, total islet insulin content in both stimulated and unstimulated islets from the same individual were compared. There was a trend towards higher insulin in the stimulated islets when data from all 10 cats were included in analysis. (p=0.06, Figure 3.4a) When males and females were evaluated separately, males showed increased insulin in the stimulated islets as compared to unstimulated controls, although the difference was not significant. (p=0.1, Figure 3.4b) In contrast, only 1 of 5 female cats showed any evidence of response to glucose challenge with the other four cats containing mildly decreased insulin in stimulated islets compared to controls. (p=0.3, Figure 3.4c) When the magnitude change in insulin content of stimulated vs control islets from males or females was compared, there was a trend for islets from males to have greater relative stimulation than those from females. (p=0.06, Figure 3.4d)

No clear patterns were identified when results of both the insulin release and islet insulin content from the same individual were compared. There was evidence of both glucose stimulated insulin release and increase in pellet insulin content for only one of the nine cats. In three cats, glucose stimulation resulted in a decrease of insulin released into the media and in the cell pellet. In all other cats there was no parallelism between the results of insulin release and pellet insulin content. (r^2=0.04, p=0.65, Figure 3.4e)

3.4 Discussion

3.4.1 Islet Isolation

Yields of purified islets vary between species. An average mouse pancreas is expected to yield approximately 300 islets, which is believed to be between 10-30% of the total islet volume.[3,9] Slightly better yields are common from human pancreases. The total number of islets per adult human pancreas is estimated to be greater than 1 x 10^6, and yields of 250-450,000 islets

(25-40%) are common.[10-12] Similar numbers of isolated islets are seen in adult pigs where yields of greater than 250,000 islet clusters are typical.[5]

In contrast, attempts to develop techniques for the isolation of large numbers of highly pure islets from the cat have been less successful. Of the two published protocols for islet isolation in the cat, neither protocol produced a total number of purified islets similar to the number of purified islets described routinely in other species.[13,14] Maeno, *et al* reported isolating less than 300 islets free of any exocrine tissue but a mean of 2200 "islet like clusters". Zini, *et al* did not quantify the number of purified islets but estimated them to represent less than 2% of the total number of islets. There are no reports quantifying the total number of islets in the feline pancreas, but the total islet mass is reported to be 3% of the pancreas.[15] Both of these investigations used the same collagenase product and a similar digestion procedure. The reasons for this disparity between cats and other species are not understood, but may involve ultrastructural anatomy of the peri-islet space, enzyme selection and delivery, connective tissue composition, or native mechanisms intended to protect the pancreas against autodigestion.

Ultrastructural investigation of the interstitial space surrounding the pancreatic islet in the cat reveal a fairly thin layer of connective tissue surrounding the islet in contrast to that described in people or rodents.[13,16] The authors of one study hypothesized that the lack of connective tissue makes it less likely that collagenase digestion of the organ can liberate large numbers of islets.[13] It is also possible that it is the collagen composition of the connective tissue fibers and not anatomical thickness that is influencing the inconsistent effect of the selected collagenase preparation in cats as compared to other species. One study described remarkable variability in the type and amount of collagen and laminin as well as the organization of connective tissue and cell adhesion molecules from the peri-islet space of dogs, people, rats, and pigs.[16] The composition of peri-islet matrix in cats has not been studied.

The yield of highly purified islets in this study is higher than what was reported previously in cats. In those reports, the major product of digestion were "islet-like clusters" which are clusters of cells that predominantly include islets with varying degrees of exocrine tissue associated with the islet.

Infusion of the pancreas with collagenase solution is often accomplished via cannulation of the pancreatic duct or the common bile duct in species other than the cat. The cat presents an anatomical challenge due to the small size of the pancreatic duct and the presence of an accessory pancreatic duct in some individuals that makes it difficult to adequately inflate the organ without careful surgical dissection. Because of the anatomical difficulties and the lack of adequate, clean surgical space in the shelter euthanasia facility, trans-capsular injection of collagenase solution after cold-storage transportation of the intact organ from the shelter to the laboratory was selected, similar to the technique used in previous studies. Trans-capsular injection of collagenase solution was effective at inflating the entire pancreas and appeared to provide consistent, homogenous digestion of the organ with little to no macroscopically un-digested tissue remaining. However, in at least one study investigating collagenase digestion of the human pancreas, it was demonstrated that collagenase infusion via the pancreatic duct was more effective at reaching the surface of the islets as compared to collagenase insufflation via trans-capsular injection.[12] If this is also the case in the feline pancreas, efficiency of digestion may be improved by infusion of enzyme through the pancreatic duct.

Islet microvasculature is unique as compared to the vasculature of the exocrine pancreas. Islets are heavily vascularized by fenestrated capillary beds to allow rapid diffusion of peptide hormones (insulin and IAPP) into circulation. Of particular interest is that islet vascular endothelium expresses alpha-1 proteinase inhibitor and islet endothelium appears to be resistant to the digestive effects of trypsin.[17] This mechanism, which is intended to be protective of the islets because of their proximity to the aggressive digestive enzymes contained in the exocrine

pancreas, may help explain some of the variability in islet yield. In fact, a common finding in our isolation procedures in cats is that many islets remain attached to exocrine tissue via one or more capillaries, and capillary remnants are commonly seen projecting from the islet surface after isolation. (Figure 3.5)

Islets were handpicked for purity in this study. Islet morphology varies tremendously within the same individual. Islets range in size from as little as 50μm to as large as several hundred micrometers in diameter. Moreover, unstained islet color is very similar to the unstained acinar tissue surrounding the islets. Because of this, it is extremely difficult to reliably identify islets based solely on morphology. Beta cells are rich in zinc and so the use of dithizone, a zinc chelating dye, provides a reversible way to positively and specifically identify islets in pancreas digestate.

3.4.2 Islet Viability

Islet viability was determined using two different assays. The LIVE/DEAD® assay is particularly useful as a non-lethal method for identifying cells with intact intracellular metabolic pathways (calcein am) as compared to non-viable cells with damaged cell membranes (ethidium). A combination of the three dimensional nature of the islet and the dramatically different intensity of fluorescence of each compound made accurate quantitative measurements using the fluorescent assay difficult. Therefore, subjective measurements of islet viability were made based on still images of several focal planes within the same islet. Using this method, viability of intact islets was estimated to be greater than 90% at 24hrs.

Release of LDH into the media was employed as an objective measurement of cell death in the system. The LDH assay was heavily affected by the presence of serum in the media. In order to measure total LDH release into the media over the five-day period, groups of islets were maintained in serum-free media for LDH assay only. Because of the concern that maintaining in

serum-free media would have a negative impact on cell viability during this time, islets were also stained with LIVE/DEAD® and compared to islets maintained in a serum-containing media. The overall viability of islet cells was estimated by LDH release to be 77% after five days in culture with serum-free media. Islets maintained in serum-free media did appear to have greater numbers of dead cells as compared to those grown in serum containing media. (Figure 3.2c-d) Therefore, it is likely that actual viability of islets when maintained in CMRL media containing 10% fetal calf serum (the media of choice) is higher than that which was calculated using the LDH assay.

Viability calculated in these experiments was of the entire islet. It is impossible to know if one cell type was less viable than others. It is well documented that freshly isolated islets undergo a change in cellular composition as a result of the isolation procedure. In particular, loss of alpha and beta cells as the result of the collagenase process and cold-ischemic time.[18] Additional experiments are required to determine which cell lines suffer the greatest losses during culture in the cat. Any change in the proportions of different cell types within the islet may alter paracrine interactions of islet components and ultimately alter islet function.[19]

3.4.3 Glucose Stimulated Insulin Production and Release

Glucose stimulated insulin release (GSIR) is routinely used as an indicator of retained islet function in freshly isolated islets from people and rodents.[3,20] Under normal physiologic conditions, insulin release in response to increased blood glucose occurs in two phases: an immediate phase in which secretory granules ready to dock release their stored insulin cargo within minutes of stimulation of the beta cell, and a sustained release phase that can continue for minutes to hours. In islets cultured from rodents, an increase in insulin release of at least two-fold over one to two hours is expected after glucose challenge.[3] The GSIR of freshly isolated feline islets in our experiments was inconsistent, with the majority of islets failing to demonstrate

a clear physiologic response upon exposure to glucose. Using a two-fold increase as a cut-off value at 24 hours, only two of eight cats showed an appropriate response.

These results suggest that our freshly isolated feline islets fail to retain their physiologic function at 24 hours post isolation. The reason for this failure of isolation could be the result of any one or a combination of the following: sub-optimal culture conditions (media, islet plating density, culture vessel) for feline pancreatic islets, excessive damage to islet structure during collagenase digestion, exposure of islets to toxic conditions (e.g. hypoxia, oxidative damage) that leave the beta cells unable to respond to glucose, inappropriate GSIR protocol for the given species, or alterations in islet paracrine signaling resulting from changes in islet cell composition.

Culture conditions recommended for human and rodent islets differ. For instance, the most commonly used media for rodent islets (RPMI) contains more glucose (10mM) as compared to the preferred media for human islets (CMRL-1066 containing 5mM glucose).[21] Additionally, the ideal supplementation of antioxidants and growth factors for pancreatic islets is not clear. Selenium supplementation has been recommended an essential element for several antioxidant enzymes. Transferrin supplementation in media can improve viability of some cell types by regulating iron delivery to the cells. Insulin-like growth factor is important for anti-apoptotic survival signaling.[21] There are no studies comparing media types or supplements for the culture of feline islets.

Pancreatic islets from several species have been successfully maintained in a variety of culture systems. Some encourage maintenance of extracellular interactions by embedding islets in matrix (collagen, elastin, gelatin, fibrin, or complex matrices such as Matrigel®) or by allowing single surface contact with a collagen coated or tissue culture treated surface.[21-26] Other applications call for maintaining the islets in a continuously rotating chamber where the islets are grown without extracellular anchoring and are free-floating in media.[27]

A wide range of different culture conditions were investigated in attempts to find conditions that provided the most ideal support for feline islets. The glucose stimulated insulin release of islets under each of these conditions was similarly inconsistent. The selected combination of CMRL-1066 with serum, antioxidants, and growth factors used in this experiment was based on review of the relevant literature relating to culture of human pancreatic islets.[21] Our preference for human islet culture conditions was based on the concept that humans and cats, but not rodents, share similar clinical and islet histopathologic pancreatic findings (beta cell sensitivity to glucose, inflammatory cytokine signaling, and islet amyloidosis) during development of disease.

The goal of a collagenase digestion is to be gentle enough to maintain healthy islets, but aggressive enough to achieve dispersion of the tissue components. Although islets remained intact and grossly normal under light microscopy, ultrastructural evaluation using electron microscopy of freshly isolated rat islets suggests the presence of major damage to the cells around the periphery of the islet and to the islet basement membrane.[28] The damage is sufficient to cause death of many of these cells. Freshly isolated rat islets were markedly deficient in alpha, delta, and PP cells which are found in the periphery of a rat islet.[28] The anatomical arrangement of the feline islet is different than that of humans and rodents. In the cat, beta cells are found largely around the periphery and alpha cells more frequently in the central region of the islet. This is in contrast to rodents in which the beta cells form the core of the islet with a higher proportion of alpha cells along the periphery of the islet, and people with alpha cells scattered throughout the islet but forming a mantle around the islet as well.[18,29] Identification of a change in distribution or location of different cell types before and after isolation of feline islets was not attempted in the current study. A preferential loss of beta cells is anticipated, given the peripheral nature of these cells in the islets. However the susceptibility to the stresses of isolation of the other islet cell types is unknown.

Another potential explanation for the lack of reliable GSIR is a toxic insult to the beta cells during the isolation process. All enzyme, media, and buffer solutions used were certified endotoxin free and were prepared new for each pancreas. The entire process of isolation includes exposure to a variety of temperatures and pH. The pancreas is chilled as quickly as possible after euthanasia of the animal and maintained on ice until ready for digestion. Cold ischemia times of 24 hours have been shown to have minimal effects on islet function in people and the cold ischemia of the feline islets was limited to less than 4 hours.[7,30] Nevertheless, increasing temperature for the collagenase digestion and then immediately chilling the digested tissue on ice likely induces substantial cell stress. Handpicking of islets requires between one and two hours per pancreas. Media is held at room temperature under room air during the hand-selection process and as such, the pH of the media is slightly higher than it would be if maintained under controlled conditions with 5% carbon dioxide. The variation in temperature, dissolved gasses, and pH undoubtedly introduces additional cellular stresses during the isolation period. Because of the expectation of cell trauma and inevitable loss of viability, islets were allowed to recover for 24 hours prior to starting any experiments. It is possible GSIR results may be different if testing was performed at the time of isolation or after an extended recovery period of several days.

The GSIR procedure used in this study is modified from traditional GSIR protocols. As described before, insulin release into the media is bi-phasic with an immediate release of insulin (within minutes) from granules ready to dock and release contents, followed by a more sustained (minutes to hours) plateau of insulin release as long as the stimulus (glucose) is present. In early experiments, little evidence of glucose responsiveness was detected from islets after two hours of stimulation. In order to account for the possibility that a failure of GSIR seen in these early experiments was simply due to a sluggish, but not absent, physiologic response we modified the protocol by extending stimulation incubation time. The longer incubation should not have any impact on the short-term insulin release but would allow additional time for delayed changes in

insulin biogenesis and trafficking to develop. It is possible that the extended incubation in a buffered salt solution and not an essential media had a negative impact on islet function.

It is well documented in people and rodent islets that the isolation process induces alterations in islet cell composition, specifically, the loss of alpha cell population. Studies in human islets have demonstrated that even a slight over-digestion of the islet results in a dramatic increase in loss of alpha cells (up to 85%).[18] Insulin release is regulated by a number of paracrine and endocrine signals in addition to glucose, principle of which is glucagon. A deficiency of glucagon has a negative impact on GSIR. The importance of glucagon in GSIR in isolated islets was demonstrated in a study using freshly isolated, alpha-cell deficient human islets in which GSIR was severely blunted without but nearly completely restored by incubation with glucagon.[18] It is possible that a similar phenomenon of altered islet paracrine signaling is responsible for the inconsistent GSIR seen in this study.

Finally, although islets are distributed throughout the pancreas and have the same overall composition, there are nevertheless differences between some islet populations. For instance, islets with a higher or lower proportion of alpha cells relative to beta cells may respond differently to glucose challenge because of glucagon's paracrine role in the islet. It has also been well described in human and rodent islets that larger islets tend to have lower insulin content and are less responsive to glucose than are smaller islets.[31,32] We cannot rule out the possibility that the small number of islets isolated could over-represent a particular population of islets that, because of their anatomical location in the pancreas or peri-islet connective tissue composition, are more likely to separate from the exocrine tissue but will not, because of the islet cell makeup and physiology, have a robust insulin response.

3.5 Conclusion

The aims of the current study were to develop techniques for the isolation of highly pure feline pancreatic islets and to demonstrate viability and functionality of freshly isolated islets. Collagenase-based digestion of cadaveric donor pancreases yielded sufficient highly pure islet tissue for many *in vitro* applications. Viability of cultured islets was good over a five day culture period and improved in serum-containing basal media. Function of islets as described by either the glucose stimulated insulin release or cell insulin content appears to vary greatly between individuals and these two measurements of function can be inconsistent within the same individual.

3.6 Acknowledgements

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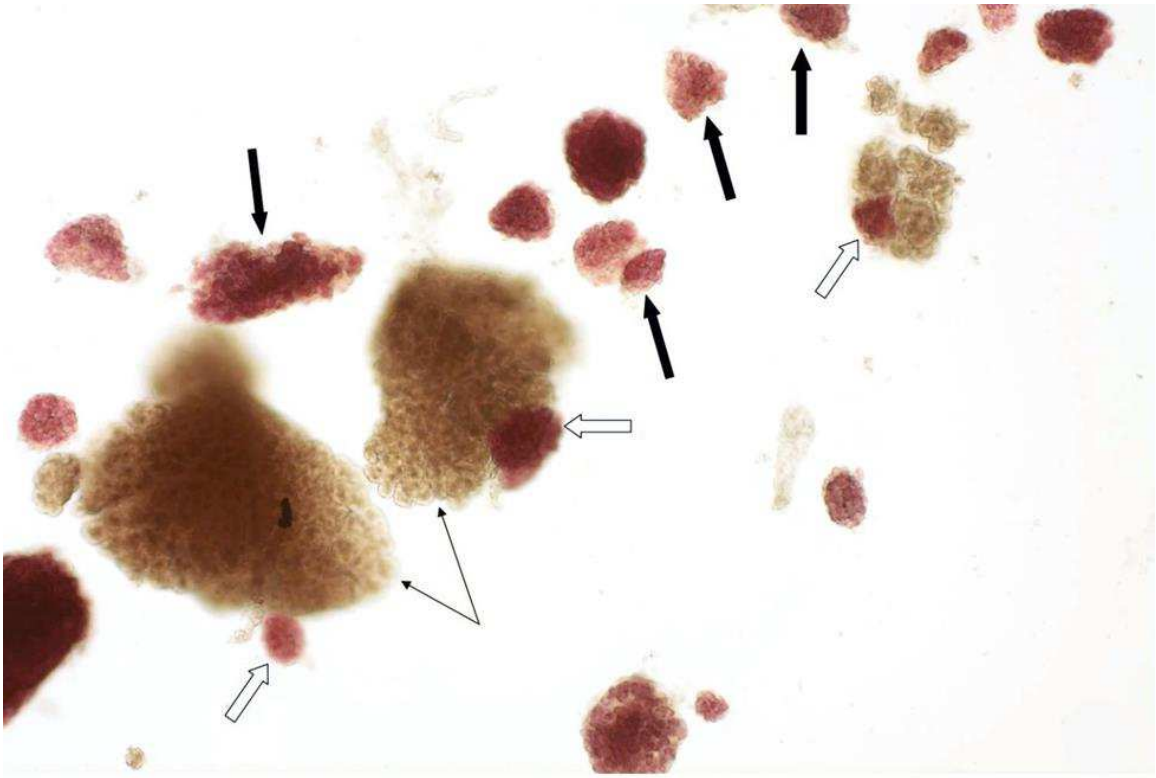

Figure 3.1. Typical semi-pure collection of islets. Free, dithizone islets (wide black arrow) are easily distinguishable from acinar tissue (thin black arrows) based on color and morphology. Several islets are still tightly adhered to acinar tissue (wide white arrows).

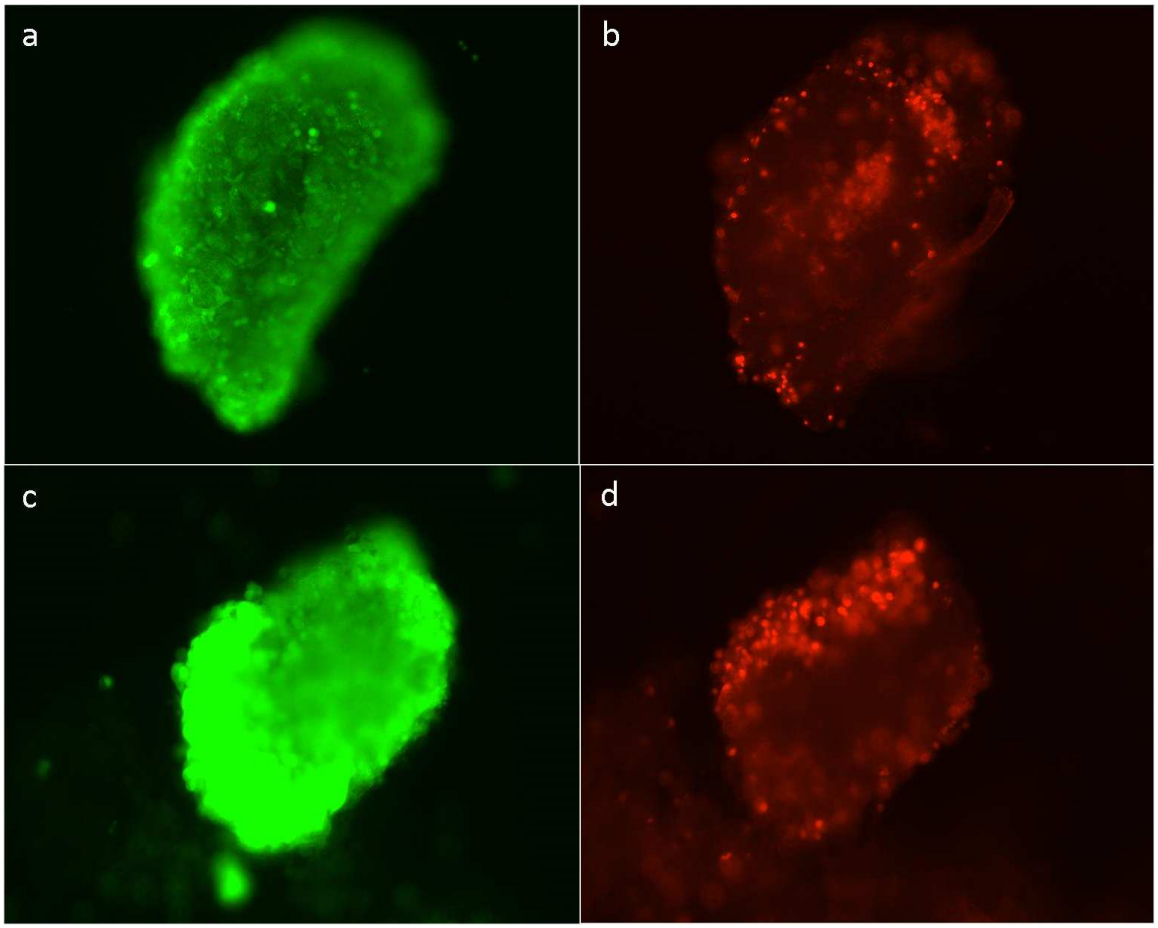

Figure 3.2. Representative images of the same islet stained with LIVE/DEAD® reagents. Green cells represent viable cells whereas individually staining red nuclei represent dead cells without intact cell membranes. Figures a-b represent islets cultured 5 days in serum-containing media. Figures c-d represent islets cultured 5 days in serum-free media.

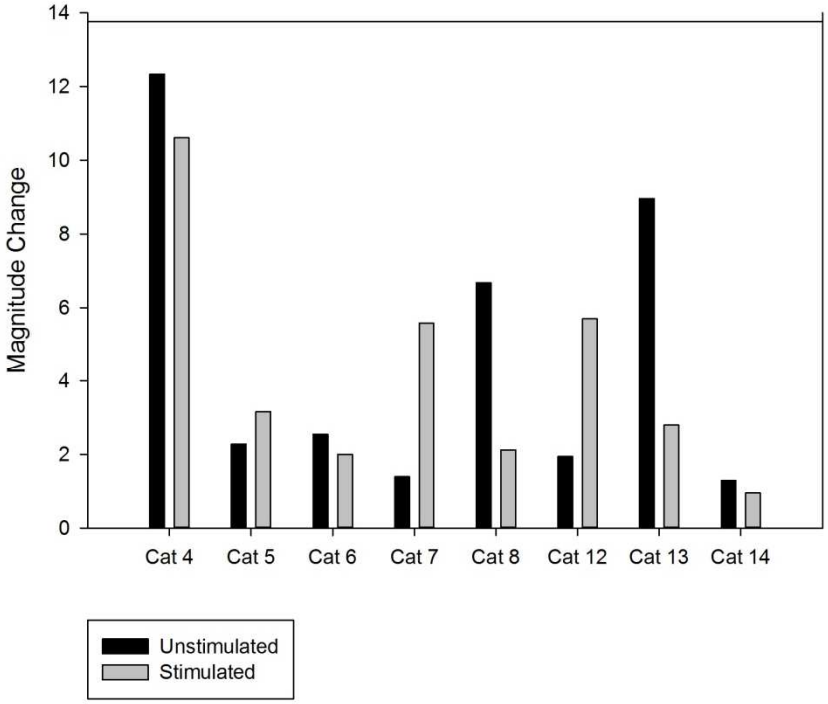

Figure 3.3. Magnitude change of insulin released into the media in stimulated vs unstimulated control islets. Resting media was replaced with media containing either high (stimulated) or low (unstimulated) glucose concentrations and islets were incubated 24 hours.

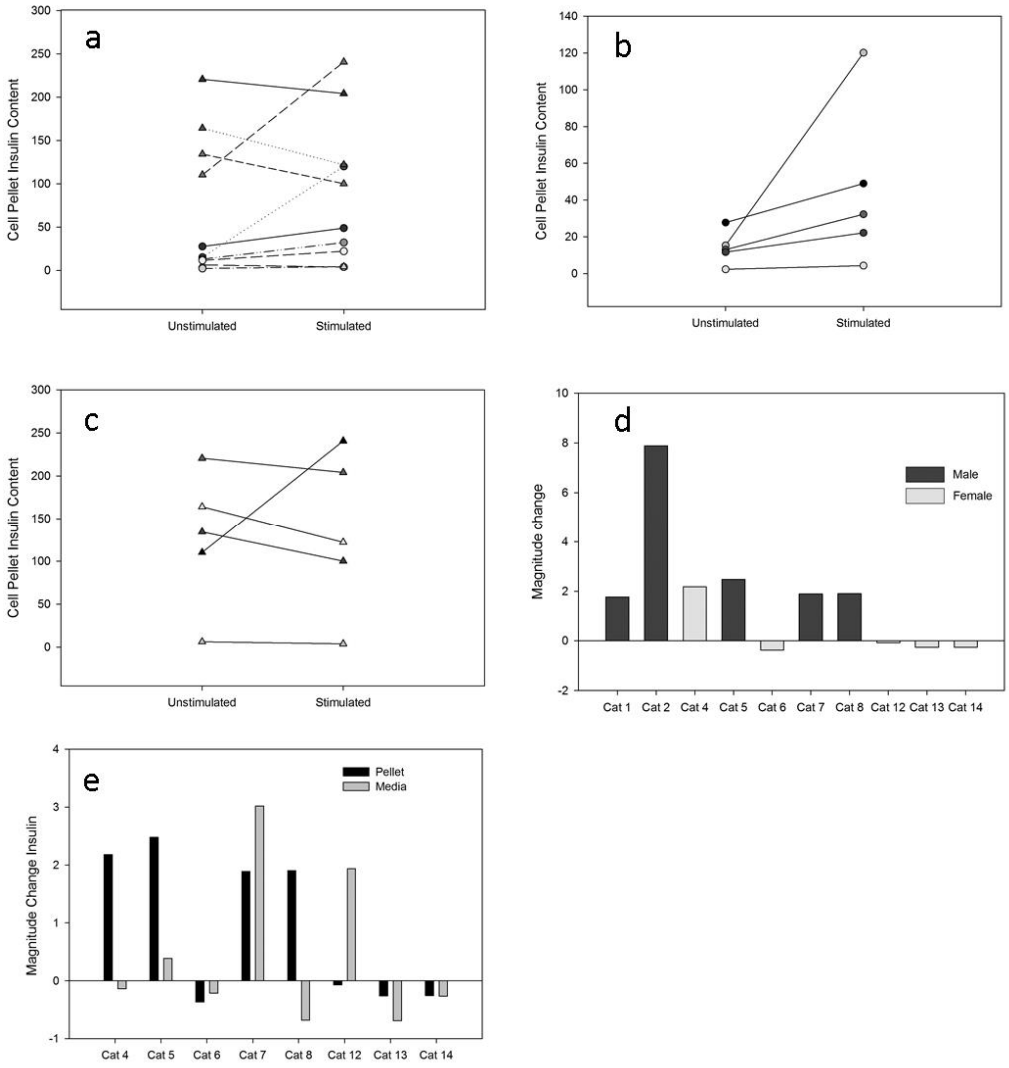

Figure 3.4. a) Paired samples of stimulated and unstimulated islets. There is a trend for higher insulin content of the cell pellets in stimulated as compared to unstimulated islets after 24hrs exposure to high glucose concentrations. (p=0.06) b) Same as Fig 4a but with data only from male cats (n=5) shown. No significant difference in insulin content of stimulated vs unstimulated islets was detected. (p=0.1) c) Same as Fig 4a but with data only from female cats (n=5) shown. No significant difference in insulin content of stimulated vs unstimulated islets was detected. (p=0.3) d) Graphical representation of the fold change in insulin content in stimulated islets from the same individual. Four of ten cats had less insulin in the stimulated cell pellets compared to

unstimulated controls. When the magnitude change in insulin content of stimulated vs control islets from males or females are compared, there is a trend for islets from males to have greater stimulation than those of females.(p=0.06) e) Comparison of the magnitude change in insulin released into the media vs insulin content of the cell pellet. Four of nine cats show discordance in released insulin content vs insulin content of cells. There was no correlation between the results of both assays. (r^2=0.04, p=0.65)

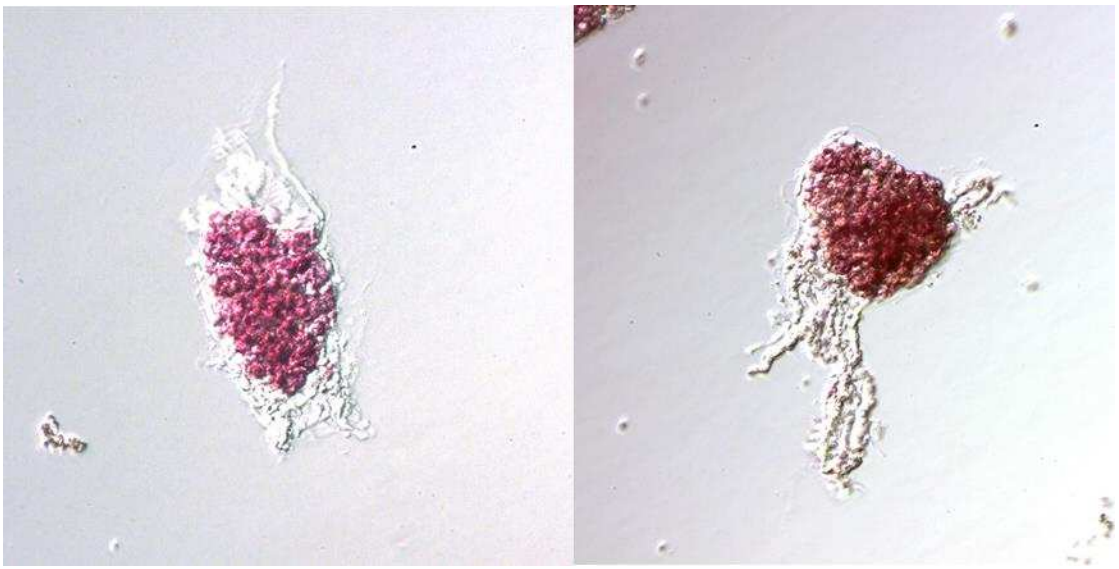

Figure 3.5. Representative dithizone stained islets with extensive islet-associated capillary remnants.

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CHAPTER 3

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CHAPTER IV

EVALUATION OF THE PROTECTIVE EFFECTS OF EXENATIDE ON FELINE ISLETS SECONDARY TO ENDOPLASMIC RETICULUM STRESS

4.1 Introduction

Feline diabetes mellitus (FDM) is the clinical manifestation of insufficient insulin necessary to maintain euglycemia. FDM develops over an extended period of time during which a period of increased insulin secretory function serves to overcome peripheral insulin resistance to maintain euglycemia. This compensatory state ultimately fails through a combination of beta cell dysfunction and beta cell loss, resulting in clinical signs of unregulated hyperglycemia. The precise mechanisms of beta cell dysfunction and loss are unknown but are thought to include oxidative stressors, development of misfolded islet associated polypeptide (IAPP), and inflammatory cytokine signaling. The presence of markers of oxidative stress, inflammation, and accumulation of IAPP in the diabetic feline pancreas was demonstrated in data supporting Aim 2.

The unfolded protein response (UPR) is a mechanism by which cells adapt to endoplasmic reticulum (ER) stress as the result of accumulated, misfolded proteins within the ER.[1] This adaptive mechanism ultimately activates the intrinsic apoptotic pathways if the stress is not ameliorated. Because of the high demand of pancreatic beta cells to produce insulin, activation of the UPR is important for maintaining beta cell function.[2] In fact, one mechanism by which IAPP is believed to induce beta cell toxicity is via excessive stimulation of the UPR.

Misfolded IAPP within the beta cells of humans and transgenic rodents is associated with increased ER stress and activation of the unfolded protein response (UPR).[3,4]

Glucagon-like peptide-1 (GLP-1) is a hormone produced by specialized enteroendocrine cells localized in the ileum of cats. Many down-stream effectors of GLP-1 receptor (GLP-1r) binding are described that collectively have a variety of metabolic effects within the beta cell.[5] Potential beneficial effects include enhanced insulin production and release in response to glucose stimulation, pro-proliferative signaling, and protection from apoptotic signals principally via activation of AKT and PKA pathways. GLP-1r signaling has been shown to inhibit apoptosis secondary to ER stress in cultures of rat beta cells and in murine insulinoma cell lines secondary to ER stress.[6-9] The availability of isolated feline islets (Chapter 3) has now made it now possible to ask a similar mechanistic question in the cat.

Based on the work in rodents and people, the goal of this study is to investigate the effect of the GLP-1 mimetic exenatide on amelioration of ER stress in freshly isolated feline pancreatic islets. We hypothesize that exenatide will reduce activation of apoptosis in pancreatic islets exposed to ER stress.

4.2 Materials and Methods

4.2.1 Islet isolation

Purified fresh feline pancreatic islets were isolated from 8 cadaveric cats being euthanized as part of a routine population control program at a local animal shelter using techniques outlined in Chapter 3, with minor modifications. All cats were young adults and included 3 neutered males, 1 spayed female and 4 intact females. After clipping hair from the abdomen, the area was briefly scrubbed and soaked with 70% isopropyl alcohol. Pancreas organs

were infused with a cold-storage solution (CoStorSol, Bridge to Life, Columbia, SC, USA) *in situ,* removed by dissection, and transported on ice. Collagenase tissue digestion (Collagenase NB-8, Serva Electrophoresis GmbH, Heidelberg, Germany) was used to liberate islets from the surrounding exocrine pancreatic tissues and islets positively stained with dithizone (Sigma, St. Louis, MO, USA) were hand-selected. Islets from individual animals were divided into five groups of roughly 60 islets each and embedded in 90µl extracellular matrix (Matrigel®, Corning®, Tewksbury, MA, USA). Islets were then left to recover 24hrs in basal media (CMRL 1066 – Corning Cellgro, Mediatech, Inc, Manassas, VA, USA, with penicillin, streptomycin, l-glutamine, and 10% fetal bovine serum – Gibco, LifeTechnologies, Grand Island, NY, USA).

4.2.2 Induction of ER stress and determination of caspase activity

After 24hrs recovery time, media was exchanged for fresh CMRL 1066 with antibiotics but without fetal bovine serum. Islets were treated for 24hrs with tunicamycin (TUN) 15µg/ml or thapsigargin (TG) 15mMol (Sigma, St. Louis, MO, USA) with or without 10nMol exenatide (Byetta®, Amylin Pharmaceuticals, San Diego, CA, USA). Tunicamycin is a synthetic antibiotic that induces ER stress in mammalian cells by blocking N-glycosylation of proteins during post-translational processing within the ER, resulting in accumulation of misfolded proteins within the endoplasmic reticulum and activation of the UPR. Thapsigargin is a non-competitive inhibitor of the sarco/endoplasmic reticulum calcium-ATPase pump (SERCA). The relative rise in cytosolic calcium and depletion of endoplasmic calcium due to dysfunction of the SERCA pump results both in induction of the intrinsic apoptotic pathway and inhibits protein processing within the ER and golgi apparatus.

Exenatide treated groups were incubated for one hour with exenatide alone prior to the addition of any other compounds to the media. Stock solutions of tunicamycin and thapsigargin were prepared by solubilizing the drugs in 100% DMSO. The final concentration of DMSO in

the treatment media after dilution was 1.5%v/v. Control islets were maintained in the same serum-free media with 1.5%v/v DMSO but without the addition of any drugs.

Activated caspase 3 was quantified using a luciferase-based luminescent assay (CaspaseGlo Caspase 3/7 assay, Promega, Madison, WI, USA). Luminescence was measured (SpectraMax M2, Molecular Devices, Sunnydale, CA, USA) and the results for each group were normalized to total double stranded DNA of the cell pellet of that group as measured by flurometric assay (Qubit® dsDNA BR Assay Kit, Life Technologies, Grand Island, NY, USA).

For results to be considered in the final statistical analysis, an increase in caspase activity of at least 50% above control had to be present in the TUN or TG only wells. The intent of including these criteria was to limit the effect of experimental variability due to inter-animal variation or baseline caspase activation during the collection process thereby allowing the effect of exenatide to be discretely examined.

4.2.3 Statistical Analysis

Data are presented as mean and standard deviation (mean ± SD). Data was evaluated using a software program (SigmaPlot, Systat Software Inc, San Jose, CA, USA) and differences between groups were tested using a two-tailed, paired t-test. Differences were considered significant when the calculated p-value was <0.05.

4.3 Results

Exposure of freshly isolated feline islets to both tunicamycin and thapsigargin resulted in increased caspase activity to approximately 1.9 times control values. Tunicamycin increased caspase activity 1.88 ± 0.14 (n=5, p=0.005) times control and thapsigargin increased caspase activity 1.92 ± 0.15 (n=4, p=0.004) times control. Treatment with exenatide alone did not change caspase activity from that of control (n=4, p=0.1). Caspase 3 activity was attenuated in islets

treated with exenatide in addition to the ER stress-inducing drug. In the case of tunicamycin treated islets, co-culture with exenatide reduced caspase 3 activity from 3141±1237 in tunicamycin treated to 2399±1299 in tunicamycin plus exenatide treated islets. (p=0.01, Figure 4.1d) Co-culture with exenatide decreased caspase 3 activity from 3141±1237 in thapsigargin treated cells to 2399±1299 in thapsigargin plus exenatide treated islets. (p=0.08, Figure 4.1c) In three experiments treatment with TUN (or TG) failed to induce an increase in caspase activity. In those samples, the unstimulated caspase activity was nearly twice the mean of controls in all other experiments and was outside the 95% confidence interval calculated for control caspase activity suggesting a problem occurred in the collection of these islets, such as contamination or excessive digestion and loss of cell integrity.

4.4 Discussion

The high metabolic rate of the pancreatic beta cell and demands on beta cell ER manufacturing capacity leave the beta cell particularly susceptible to stress associated with ER dysfunction. The role of ER stress in beta cell dysfunction has been well described in people and rodents, but has not been described in cats. In this study, ER stress and caspase activation was induced in freshly isolated feline islets via two separate mechanisms. Co-treatment with the GLP-1r agonist exenatide resulted in attenuation of caspase activity suggesting that exenatide may protect pancreatic islets from apoptosis secondary to ER stress.

Caspase 3 activity was selected as an outcome variable because overstimulation of the UPR secondary to ER stress ultimately result in caspase 3 activation and apoptosis. Additionally, the effect of GLP-1r activation involves multiple pathways, several of which culminate in inhibition of caspase 3 activation. Therefore, caspase 3 activity represented a convergence of both GLP-1 signaling and ER stress. In order to better understand the specific mechanism of the protective effects of exenatide, the various components of the UPR and GLP-1 signaling pathway

would need to be investigated. Of particular interest is phosphorylated PERK and eIF2α as these have been shown to be central to the effects of GLP-1 activity in human beta cells. Additionally, characterization of changes in the Bcl-2 family of proteins under ER stress and after treatment with exenatide would provide insight into activation of the intrinsic pathway of apoptosis. (Figure 4.2)

Three of the eight cats failed to show adequate caspase activation following TUN or TG treatment. All three of these cats also had high baseline caspase activity in the control islets. High caspase 3 activity in control islets might be attributed to several potential causes. Islets were allowed a 24 hour recovery period post isolation to recover from the stress of isolation. Even with this recovery period, it is likely that there will be some degree of cell stress related to the isolation and culture procedures. The process of isolation involves multiple changes in temperature and several hours cold-ischemia time, exposure to collagenase enzyme which can disrupt cell integrity, and exposure to agents that may be associated with oxidative stress to the cells (as discussed in Chapter 3). Contamination with a bacterial agent or source of endotoxin would result in large increases in caspase 3 activity. Additionally, donor cats for this experiment were sourced from the local shelter and as such they have a varied genetic background, unknown age, and unknown health status (beyond appearing healthy to shelter staff prior to euthanasia).

In order to limit the effects of various growth factors found in fetal calf serum, serum free media was used for the 24 hour incubation with tunicamycin and thapsigargin. The removal of protective effects associated with serum (growth factors, albumin, transferrin) may be associated with a mild increase in caspase activity. The effects of serum-free versus serum-containing media on caspase 3 activation were not investigated.

Although not significant, there appeared to be a modest increase in caspase 3 activity in islets treated with exenatide alone. Meta-cresol is a preservative used in the formulation of

Byetta®. This compound is commonly used in cosmetics and pharmaceuticals and is well studied. Toxic concentrations of m-cresol vary widely depending on the cell type and response investigated (lethality, inhibition of growth, inflammatory cytokine production). In one study evaluating the toxicity of m-cresol on rat isolated hepatocytes, a dose-dependent inhibition of mitochondrial respiration was seen at concentrations as low as 0.3nmol/µg protein.[10] Based on total protein content of similarly isolated and cultured islets used in previous experiments, we estimate islets in our experiments were exposed to between 0.3 and 0.5nmol/µg protein of the sample. This would suggest that the dose of m-cresol could produce a negative impact on mitochondrial respiration. In contrast, other studies using cell lines from fish or mice have only shown measurable toxicity at concentrations at least 100x the doses to which the islets in our experiments were exposed.[11,12]

DMSO is also a known toxin to mammalian cells. Although it is commonly used as a cryopreservative in pancreatic islets at a concentration of 10%, it is unclear what concentration of DMSO will be toxic under extended culture at 37deg celsius.[13,14] Islets from all treatment groups in our experiments were exposed to 1.5%v/v DMSO to control for the potential negative effects of DMSO at that concentration.

Finally, the concentration of exenatide used in this experiment was 10nMol, which is a greater concentration than what has been described in plasma of cats administered exenatide. This dose was extrapolated from the literature in experiments using isolated islets and cell cultures in other species.[15-18] The pharmacokinetics of exenatide extended release formulation (Bydureon®, Amylin Pharmaceuticals, San Diego, CA, USA) in cats have been described.[19] A mean peak plasma exenatide concentration of 0.27ng/ml (64.5pMol) was achieved after a single dose of 0.13mg/kg administered subcutaneously. This dose resulted in an increase in the area under the curve for insulin following glucose stimulation and decreased glucagon concentration during hyperglycemic clamp procedure. The pharmacokinetic profile of the twice-daily exenatide

formulation has also been investigated. In that study, administration of a single subcutaneous dose of 1μg/kg exenatide produced a mean peak plasma concentration of 3.0ng/ml (716pMol) with similar pharmacodynamic effects.[20] Therefore, the dose of exenatide used in the current study was roughly 155 times the theoretical *in vivo* exposure for extended release exenatide and 14 times that seen with the twice daily formulation. Additional investigations using varying drug doses in cultured islets are necessary to evaluate if similar responses are seen to more clinically achievable doses.

4.5 Conclusion

The purpose of this experiment was to describe the effects of the GLP-1 mimetic exenatide on caspase 3 activity in freshly isolated feline islets exposed to ER stress. Our results suggest that exenatide may have a protective effect against caspase 3 activation (and secondarily, apoptosis) in islets exposed to severe ER stress secondary to treatment with thapsigargin or tunicamycin. These findings suggest one potential mechanism for beta cell protective effects of exenatide on feline islets.

4.6 Acknowledgements

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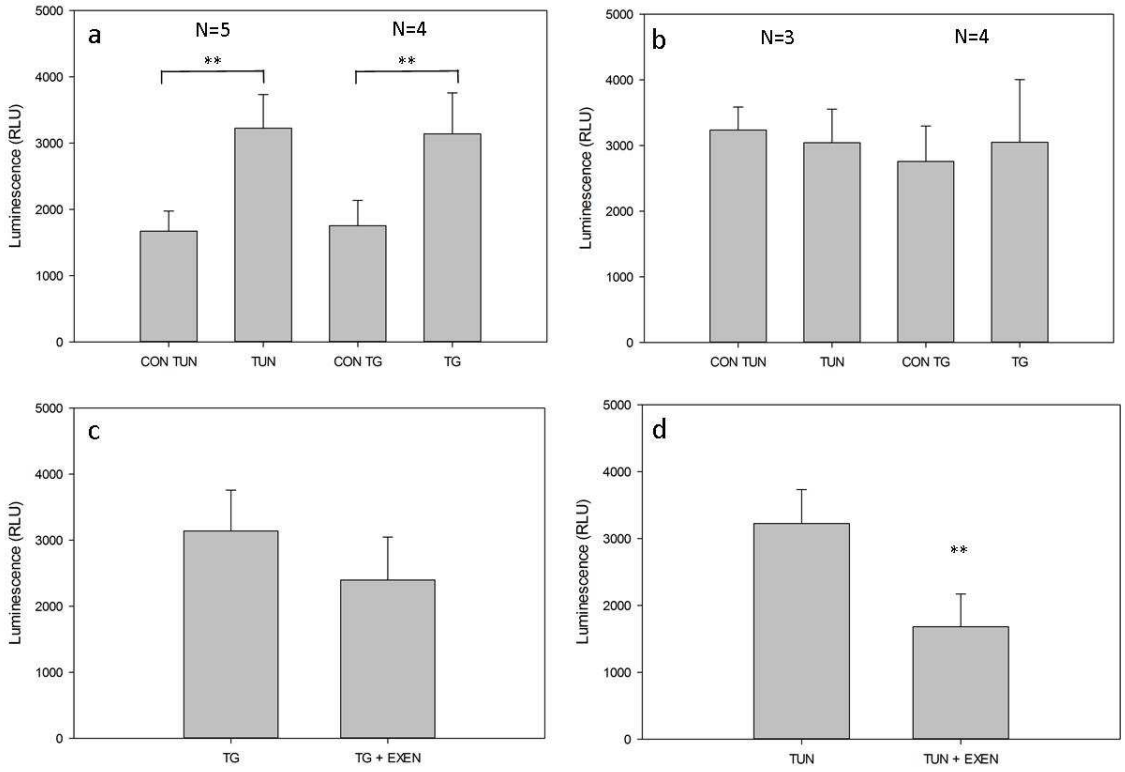

Figure 4.1. a. Caspase activity in untreated, tunicamycin treated, and thapsigargin treated islets. b. Caspase activity in data excluded due to failure of tunicamycin or thapsigargin to stimulate caspase activity. Compared to the data in Panel A, the baseline caspase activity in the untreated control is significantly (p=0.02) higher suggesting either activation during collection and processing or variability due to random source animals. c. Caspase activity in thapsigargin and thapsigargin with exenatide treated groups. There was no difference between the groups. (n= 4, p=0.08). d. Exenatide treatment in addition to tunicamycin was associated with a decrease in caspase activity (n=5, p=0.01).

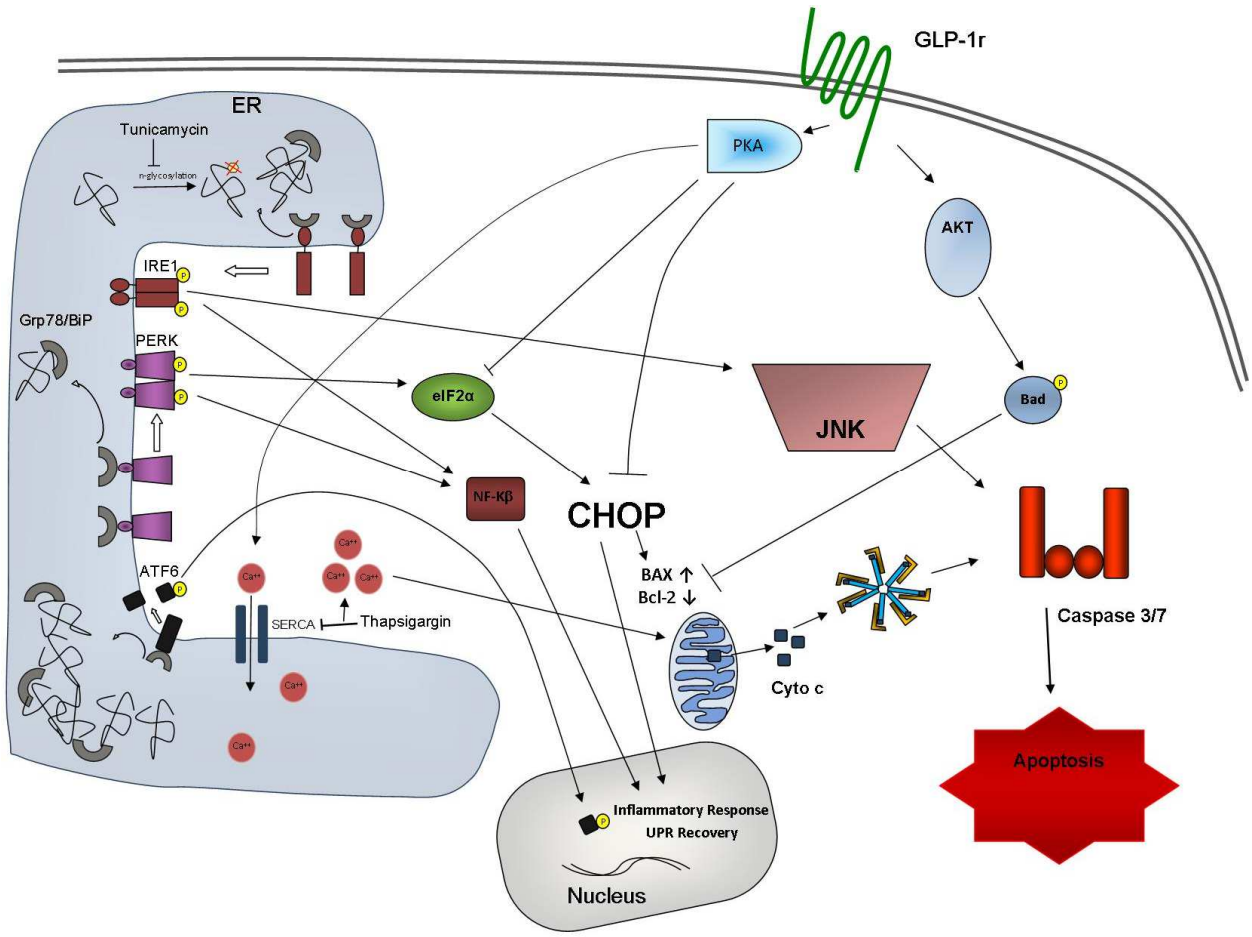

Figure 4.2. There are three canonical branches of the UPR. Accumulation of misfolded or unfolded proteins leads to disassociation of GRP78 from UPR mediating proteins, allowing dimerization and phosphorylation of IRE1 and PERK and phosphorylation of ATF6. IRE1 activation will in turn up-regulate the activity of XBP1s (not shown) which serves as a transcription factor to promote chaperone protein production (such as BiP). Excessive activation of IRE1 also activates the JNK pathway leading to apoptosis. PERK phosphorylates eIF2α which up regulates the activity of CHOP. CHOP functions include induction of mitochondrial stress (and activation of the intrinsic pathway to apoptosis) and serving as a transcriptional regulator of several pro-inflammatory and pro-apoptotic products. ATF6 phosphorylation allows for translocation of the cytoplasmic domain of the protein to the nucleus where it serves as a transcription factor for, among other things, several chaperone and UPR-related proteins.

Thapsigargin inhibits the function of the SERCA pump, thereby depleting ER calcium stores. Decreased ER calcium causes ER swelling and promotes misfolding while increasing cytoplasmic calcium leads to activation of the intrinsic apoptotic pathway. Tunicamycin inhibits n-glycosylation of proteins, leading to misfolding and accumulation of these proteins within the ER. GLP-1r signaling is anti-apoptotic via several mechanisms: direct inhibition of eIF2α, activation of the SERCA pump, inhibition of CHOP, and inhibiting pro-apoptotic Bcl-2 family proteins via phosphorylation of Bad.

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CHAPTER 4

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CHAPTER V

EVALUATION OF EXENATIDE AS AN ADJUNCTIVE TREATMENT FOR DIABETES MELLITUS IN THE CAT

5.1 Introduction

The arsenal of treatments available for feline diabetes mellitus is limited and remains centered on the eventual requirement of parenteral insulin therapy. Advances in our understanding of the unique glucoregulatory physiology and nutritional requirements of cats as well as the addition of new insulin formulations have made for significant improvements in treatment outcomes of diabetic cats. However, these treatments fall short in comprehensively addressing the underlying pathophysiology of beta cell dysfunction.

Feline diabetes mellitus is a clinical disease resulting from insufficient insulin activity to maintain normoglycemia. The reasons for this loss of activity are related both to alterations in insulin responsiveness in insulin-sensitive tissues as well as loss of insulin production by the pancreatic beta cells in amounts sufficient to match peripheral insulin sensitivity.[1] Peripheral insulin sensitivity is influenced by factors such as diet, exercise, total body adiposity, and endocrinopathies affecting insulin receptor regulation and function (e.g. hypercortisolism, hypothyroidism).[2,3]

The mechanisms of beta cell loss or dysfunction in the cat are less clear. Proposed mechanisms for loss of functional beta cell mass include direct gluco-lipotoxicity, oxidative stress, inflammatory cytokine signaling, and accumulation of misfolded islet associated (or islet

amyloid) polypeptide (IAPP).[4-8] As with Type 2 diabetes mellitus in people, feline diabetes appears to be associated with a gradual loss of beta cell function combined with peripheral insulin resistance that appears to occur over an extended period of weeks or even years. The remaining beta cell population attempts to compensate by resisting apoptosis and proliferating under the regulation of hormones such as glucagon-like polypeptide-1, insulin, and prolactin.[9]

The "incretin effect" refers to the augmented insulin response to enteral nutrients as compared to an equivalent parenteral dose. Incretin hormones are produced by specialized endocrine cells within the gut in response to luminal nutrient exposure. The two most notable incretins are glucagon-like polypeptide-1 (GLP-1) and gastric inhibitory peptide (GIP). These incretin hormones have some overlapping physiologic functions including promoting insulin production and release in response to glucose, inhibiting apoptosis in beta cells, and inducing satiety signals in the brain.[10] GLP-1 appears to have more broad-reaching effects on the beta cell to include promotion of beta cell proliferation and neogenesis.[11-13] Incretin hormones are substrates for dipeptidyl peptidase-4 (DPP-4) which is widely expressed in all tissues. DPP-4 activity in the intestinal capillary bed and portal circulation rapidly degrade active GLP-1 and GIP into inactive peptides and it is estimated that only 25% of hormone reaches portal circulation and only 10% reaches the vena cava.[14]

Because feline diabetes is the result of a relative inadequacy of beta cell function but not necessarily an absolutely loss of beta cell numbers, the therapeutic use of incretin hormones for their beta cell insulinotropic, cytoprotective, and cytoproliferative effects is appealing. There are two primary approaches to incretin therapy in the clinical setting. One is to inhibit DPP-4 activity, thereby increasing endogenous incretin hormone concentrations. Another approach is to develop incretin "mimetics" that have the same biological activity but are resistant to DPP-4 degradation.

The oral DPP-4 inhibitor sitagliptin (Januvia®, Merck) has been evaluated in a small number of cats.[15,16] Although one study detected a modest effect on post-prandial insulin concentration in healthy cats after administration of sitagliptin, the second study failed to detect any effect. .

Exenatide is a synthetic analog of a peptide originally discovered in *Heloderma suspectum* saliva, which shares a 35% sequence homology to GLP-1, has nearly identical biological effects but is a poor substrate for DPP-4. Exenatide is marketed for human diabetic patients under the trade name Byetta® as a twice-daily injection and Bydureon® as an extended-release formulation. There is an extensive body of literature on the clinical effects of exenatide in human Type 2 diabetics, but the body of literature describing the clinical application in veterinary patients is still limited. The pharmacokinetics and pharmacodynamics for both the short acting and extended release formulations of exenatide have been studied in healthy cats.[16-19] Both formulations were associated with an increase in the area under the curve (AUC) for insulin following glucose stimulation and showed a similarly high safety margin.

Based on the documented insulinotropic effect and theoretical cytoprotective and cytoproliferative benefits of exenatide in cats, the aim of this clinical trial was to evaluate the effect of exenatide as an adjunctive treatment for cats with newly diagnosed diabetes mellitus. Our study hypothesis was that the addition of exenatide to standard insulin therapy will be associated with better glycemic control as determined by serum fructosamine, decreased insulin dose, and reduction in body weight over the 12 week study period.

5.2 Materials and Methods

5.2.1 Study Population and Inclusion Criteria

Cats referred to the Boren Veterinary Medical Teaching Hospital at Oklahoma State University with a recent diagnosis of feline diabetes mellitus were enrolled during two separate

periods due to a period of unavailability of the insulin formulation used in this study. Cats were enrolled between September, 2008 and June, 2010 and again between July, 2014 and March, 2015. A diagnosis of diabetes mellitus was made based on a combination of increased serum glucose (greater than 180mg/dL), glucosuria and increased serum fructosamine. Patients treated with exogenous insulin within 30 days prior to admission were excluded. Cats with concurrent, systemic disease not directly related to their diabetes such as hypertension, neoplasia, clinical pancreatitis, or endocrinopathies (e.g. hyperthyroidism, hypersomatotropism) were also excluded. The study was approved by the Oklahoma State University Institutional Animal Care and Use Committee.

5.2.2 Diagnostic testing

Upon admission to the study, all cats had comprehensive in-house diagnostics to include serum biochemistry panel, complete blood count, total serum thyroxine concentration, urinalysis, aerobic urine culture, and non-invasive systolic blood pressure. In addition, feline pancreatic lipase immunoreactivity (fPLI), tripsin-like immunoactivity (TLI), cobalamin, and folate were measured. In three of the cats, a brief ultrasound of the cranial abdomen, specifically evaluating the pancreas, duodenum, and biliary tree was performed.

5.2.3 Study Design

Patients were randomly assigned to one of two groups in a double-blinded, placebo-controlled study design. Group A cats were treated with porcine zinc insulin (Vetsulin®, Intervet) twice daily (q12hr) at an initial dose of 0.25IU/kg and exenatide (Byetta®, Amylin Pharmaceuticals) twice daily (q12hr) at a dose of 2mcg/kg. Group B cats were treated with the same insulin formulation and dose but were administered sterile saline in an equivalent volume to that of group A exenatide treatment. Diet was not controlled and owners were free to continue

feeding the patient's current diet, however, a therapeutic diet designed for the diabetic cat was available if owner's elected to modify the patient's diet.[a]

Patients were examined every two weeks over a 12-week study period. At each visit, a complete physical exam and in-hospital serum blood glucose curve were performed starting at the time of morning insulin dose and continuing for 4-6 hours after the nadir blood glucose was identified (8-12 hour total duration per curve). Blood glucose was measured with the AlphaTRAK® (Abbott Animal Health, Illinois, USA) glucometer using capillary blood obtained by ear pinna prick. Serum fructosamine was evaluated at least once monthly, but could be requested at any recheck visit to assist in making adjustments in insulin dose. Owners were asked to log any changes in the pet's behavior daily and immediately report any gastrointestinal signs of vomiting, diarrhea, or anorexia.

Insulin was initially dosed at 0.25IU/kg rounded to the nearest 0.5IU using 0.3cc U-40 syringes to facilitate accurate dosing of the small volumes of drug prescribed. After each visit the insulin dose was adjusted based on clinical signs, blood glucose curve, and serum fructosamine. (Figure 5.1) Adequate glycemic control was defined as a reduction in clinical signs of polyuria and polyphagia along with serum fructosamine less than 550µMol and a glucose curve nadir between 100 and 200mg/dl. Insulin doses were increased by 25% or 0.5IU in cases of inadequate control and decreased by 25% with evidence of over-control (nadir glucose <100mg/dl, clinical signs of hypoglycemia at home).

At the end of the study period, fructosamine, PLI, and a blood glucose curve were measured. Study drug was discontinued after the final visit at week 12 but insulin treatment continued with porcine zinc insulin on a twice-daily schedule.

5.2.4 Statistical Analysis

Data are presented as mean and standard deviation (mean ± SD). Data was evaluated using a software program (SigmaPlot, Systat Software Inc, San Jose, CA, USA). Results for weight, fructosamine, fPLI, and insulin dose were evaluated using a two-way repeated measures ANOVA with Tukey Pairwise Multiple Comparison post-hoc analysis. Differences were considered significant when the calculated p-value was <0.05. A post-hoc power and sample size analysis was performed for the outcome variables of ending fructosamine and insulin dose. For this analysis, the values of alpha and beta were assumed to be 0.05 and 0.20 respectively.

5.3 Results

Thirteen eligible cats were presented during the study period. A diagnosis of diabetes mellitus was confirmed in all 13 patients. In one case, a diagnosis of hypertension was made on initial examination (systolic blood pressure 190mmHg) prior to any additional diagnostics being completed. The owner did not return for a follow-up examination to verify the diagnosis and so the patient was not enrolled. Twelve cats were enrolled in the study including 6 castrated males and 6 spayed females aged 10.7 ± 2.2 years. (Figure 5.2)

Two cats failed to complete the entire study protocol. One cat was withdrawn by his owner due to marked anorexia that was attributed to study drug. A five day drug holiday was recommended during which time the patient's appetite and attitude returned to normal. The drug was re-started for two days during which time the patient again became profoundly anorexic and his owner elected to remove him from the study. A second cat failed to show adequate response to insulin, despite a continuously escalating insulin dose. Although she had no clinical signs associated with acromegaly, an elevated IGF-1 of 257nmol/L (reference range = 12-92nmol/L) was consistent with a diagnosis of acromegaly. A diagnosis of a concurrent endocrinopathy necessitated removal of that patient from the study.

A summary of clinical pathologic findings is presented in Figure 5.3. Random treatment group assignments resulted in equal numbers of spayed females and neutered males in each treatment group. There was no difference between the exenatide or saline groups regarding starting or ending body weights. (p=0.84, 0.99 respectively). Although five of six cats in the exenatide group lost weight (mean weight change -0.19 ± 0.32kg) compared to only 2/4 control cats (mean weight change 0.025 ± 0.173kg), the difference was not significant (saline group p=0.86, exenatide group p=0.13).

Serum fructosamine concentration was not different between exenatide (635.7 ± 114.1) and saline treated controls (569.6 ± 111.6) at the start of the study period (p=0.81). Fructosamine was also not significantly different between the two groups after 12 weeks. (exenatide: 557.2 ± 120.06, saline: 499.8 ± 97.52, p=0.39) Mean fructosamine concentrations decreased within each group over 12 weeks, however, the differences between starting and ending means within groups were not significant (exenatide group p=0.12, saline group p=0.07)

Pancreatic lipase activity was similar between both exenatide treated and saline treated cats at the beginning and end of the study. (p=0.53, 0.38 respectively) Mean serum PLI did decrease slightly in both groups during treatment. In the exenatide group, PLI decreased from 10.10 ± 9.21 to 9.20 ± 10.43, but this change was not significant. (p=0.51) In the saline treated group, PLI decreased from 7.62 ± 4.14 to 3.05 ± 1.64, but this change was also not significant. (p=0.46)

Initial insulin dose was assigned based on body weight. Mean insulin dose (IU/kg) increased in both groups (0.54 ± 0.20 in control and 0.44 ± 0.27 in exenatide treated cats) and the difference in dose between groups was not significant at 12 weeks. (p=0.4)

The results of the post-hoc sample size analysis suggests that 45 cats in each group would be required to detect a difference in serum fructosamine and at least 70 cats in each group would

be needed to detect a difference in insulin dose requirements. Limited resources and difficulty recruiting naïve diabetic cats for the study made enrolling additional patients unfeasible.

5.4 Discussion

The aim of this study was to evaluate the utility of the addition of an incretin mimetic to standard insulin therapy. We hypothesized that the addition of a GLP-1 mimetic to standard insulin therapy would improve diabetic control resulting in lower average serum glucose and consequently lower fructosamine concentration, weight loss, and reduce the requirement for exogenous insulin.

One of the 12 cats enrolled in the study experienced an adverse event attributed to the drug. In that case, the patient was withdrawn by the owner after a report of severe anorexia that resolved upon removal of the drug and returned when drug was re-introduced at full dose. In people, it is recommended that a smaller dose be used during the first month and then increased for the duration of therapy to decrease the incidence of gastrointestinal side-effects.[20] Based on this recommendation, it is possible the drug could have been re-introduced at a reduced dose for several weeks and then increased the dose if no anorexia was seen.

Exenatide, like GLP-1, has an activating effect on the satiety center of the brain. In clinical trials, exenatide (Byetta®) was associated with between 1 and 3% incidence of decreased appetite in people. Gastrointestinal side effects including vomiting, diarrhea, and nausea occur in as many as 41% of people taking the drug.[21] In a recent meta-analysis, exenatide was found to correlate to a weight loss of 1.37kg (95% CI: -2.22kg, -0.52kg) in people.[22] Feline diabetes mellitus is frequently diagnosed in obese cats and weight reduction is associated with increased insulin sensitivity. Therefore, weight loss may be a positive result of increased satiety signals. Not all diabetic cats, however, are overweight, and in the case of an excessively thin cat, weight loss would be detrimental. Weight loss occurred in five of 6 cats treated with exenatide. One cat

from the exenatide group gained 10% of his body mass during the 12 weeks, however this cat began the study at a very low body condition and was still thin upon completion of the study. Weight loss in the remaining five exenatide treated cats ranged from 2.6-10.9% of initial body weight.

Our initial insulin dose was conservative and dose increases were expected within the first weeks of therapy. At the end of the 12-week study, mean insulin dose between the two groups was not different. If exenatide had improved glycemic control as hypothesized, we would have expected an insulin-sparing effect of the drug and a lower mean dose of insulin required to maintain euglycemia. This finding suggests that exenatide did not allow for a lower insulin dose and, therefore, did not result in improved glycemic control.

A black-box warning from the United States Food and Drug Administration was issued for Byetta® in 2008 after 36 cases of necrotizing hemorrhagic pancreatitis were associated with exenatide use in people between 2007 and 2008. Multiple meta-analyses performed since that warning was issued have failed to identify an association with exenatide use and increased risk of pancreatitis in people. However, because of the historical concern, serum feline pancreatic lipase immunoactivity was used as a marker for pancreatitis in our patients. Using a laboratory-defined cut-off of 3.5µg/L for normal PLI concentration, only two of 10 cats had normal PLI at all time-points. Serum PLI decreased over the 12 weeks in nearly all cats, regardless of treatment group, and there was no significant difference seen in PLI concentrations in cats treated with exenatide as compared to saline control. This data is consistent with published literature suggesting pancreatitis is a common feature in diabetic cats. It is important to note that PLI has a sensitivity of only 54% and a specificity of 67% in the diagnosis of chronic feline pancreatitis.[23,24] It is unknown if the elevated PLI values seen in cats in this study are truly associated with clinically relevant pancreatitis. Patients were observed closely by owners for signs of pancreatitis (anorexia, abdominal discomfort, vomiting) and any potential signs were reported. In the single

patient with a report of anorexia, repeat fPLI values declined, suggesting that pancreatitis was less likely the cause of the episode.

We did not control for diet in this study and variations in diet are likely to confound our data. The importance of carbohydrate-restricted diets in the treatment of diabetic cats has been demonstrated in several studies.[25-27] Carbohydrate restriction is associated with a more modest postprandial spike in blood glucose and lessens the demands on already taxed beta cells. Diets low in carbohydrates and high in protein are associated with weight loss while still maintaining lean body mass, both of which are associated with improved insulin sensitivity. Owners of patients in our study were counseled as to the importance of diet and suggestions for appropriate diabetic diets were made. Where owners elected, a therapeutic diet designed for diabetic cats was available. It is impossible to determine what role, if any, diet played in this study because of the small number of cats and the lack of accurate diet history.

As discussed, feline diabetes is an insidious disease characterized by a very gradual decline in beta cell function and ability to produce insulin in amounts sufficient to maintain normoglycemia. It is therefore likely that most cats have some degree of beta cell function at the time of diagnosis. Because of this, we sought a population of diabetic cats (newly diagnosed) most likely to still have functional beta cell mass remaining. The unpredictable and variable nature of beta cell loss over time combined with cat behavior, owner attentiveness, and other comorbidities means that even the classification of "newly diagnosed diabetic cats" still represents mixed population of disease. Much larger sample sizes may allow the ability to stratify patients more effectively and to identify specific subsets of patients more or less likely to respond to treatment.

Finally, a major challenge for treating a diabetic cat is defining a "well controlled diabetic." Serum fructosamine, blood glucose curves, clinical signs reported by the owner, and

changes in body condition do not always correlate well with each other in the clinical setting. The clinical significance of a change in serum fructosamine is small compared to the subjective report of reduced PU/PD or improved interaction with family. Therefore, just because a serum fructosamine value is "less than ideal" does not mean the patient may not be adequately controlled if all other data suggests adequate control. In this study, we selected serum fructosamine and insulin dose at 12 weeks as our primary, objective outcome variables most likely to correspond to adequate disease control. A more nuanced definition of diabetic control would require a much larger data set with multivariate analysis along with subjective evaluation of individual patients to determine if there may be clinical benefits to the use of GLP-1 mimetics that go beyond a single serum chemistry value.

5.5 Conclusion

The addition of exenatide to traditional insulin therapy was not associated with any significant changes in body weight, serum fructosamine, serum PLI, or exogenous insulin dose. However, our ability to detect differences was severely limited by the small sample size. Our post hoc power analysis suggests that a much larger group of cast would be required to detect the fairly small changes in fructosamine and insulin dose detected in this study. Because of these modest changes in outcome variables observed, additional value may be obtained by including subjective outcomes such as owner perceptions of diabetic control.

3.6 Acknowledgements

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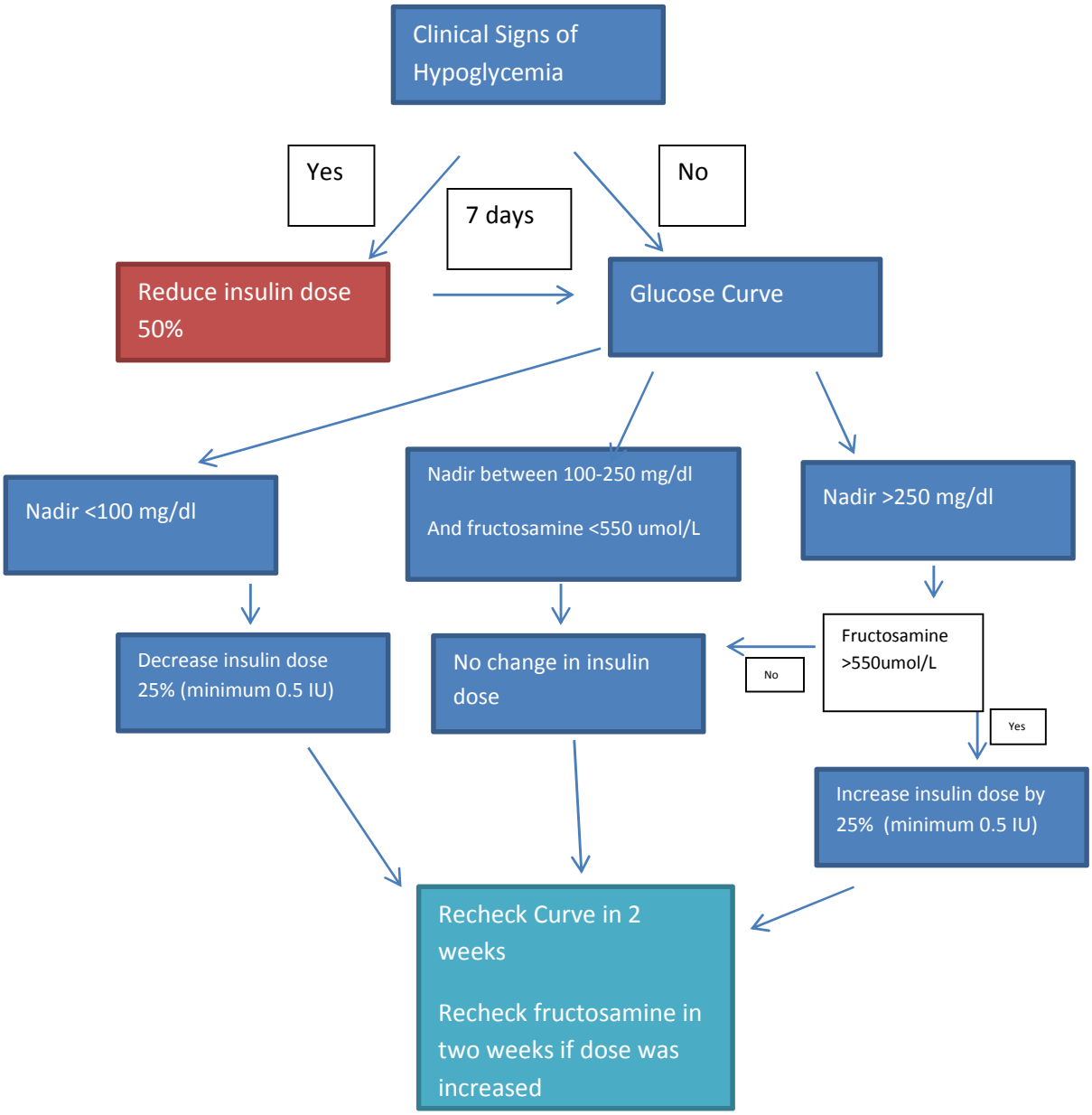

Figure 5.1. Decision making algorhythm for adjusting dose at each evaluation.

Patient	Age (yr)	Sex	Weight (kg)	BCS	Treatment Group
1	9	SF	5.04	7	Ex
2	13	MN	4.58	4	Ex
3	13	SF	3.93	4	Ex
4	9	SF	4.28	4	Ex
5	10	MN	6.5	8	Ex
6	6	MN	4.26	3	Ex
7	14	MN	3.9	3	Ex
8	10	MN	3.05	3	Sal
9	11	SF	7.0	9	Sal
10	10	MN	4.7	3	Sal
11	11	SF	3.5	3	Sal
12	12	SF	6.41	8	Sal

Figure 5.2. Summary of signalment, body weight, and body condition score along with treatment group assignment. Patients seven and twelve did not complete the 12-week study period. Body condition scores are assigned based on a nine-point scale, with 9/9 being morbidly obese. (Ex=exenatide, Sal=saline, BCS=body condition score, SF=spayed female, MN=male neutered)

		Sex	Age (Years)	Weight (kg)	Fructosamine (µmol/L)	Pancreatic Lipase Immunoreactivity (PLI) (µg/L)	Insulin Dose (IU/kg)
Time 0 weeks	Control n=5	MN=2 SF=3	10.80 ± 0.84	4.90 ± 1.09	569.6 ± 111.6	7.62 ± 4.14	0.31 ± 0.02
	Exenatide n=7	MN=4 SF=3	10.60 ± 2.88	4.67 ± 1.74	635.7 ± 114.1	10.10 ± 9.21	0.27 ± 0.06
Time 12 weeks	Control n=4	MN=2 SF=2	10.50 ± 0.58	4.53 ± 1.6	499.8 ± 97.52	3.05 ± 1.64	0.54 ± 0.20
	Exenatide n=6	MN=3 SF=3	11.30 ± 2.25	4.55 ± 1.1	557.2 ± 120.1	9.20 ± 10.43†	0.44 ± 0.27

Figure 5.3. Data summary for all cats. 12 cats were enrolled, but only 10 cats completed all 12 weeks of the study. No significant differences were found between groups. See text for individual p-values. All values are mean ± standard deviation. †n=5 used in analysis due to missing data point

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CHAPTER 5

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CHAPTER VI

DISCUSSION

Diabetes mellitus is one of the most common endocrinopathies seen in cats. It is the result of insufficient insulin activity within insulin-sensitive tissues to maintain euglycemia and is the result of both changes in peripheral insulin sensitivity and lack of adequate functional beta cell mass. The loss of beta cell function occurs gradually over a period of months and years, and mechanisms of loss are incompletely understood in the cat.

The purpose of the work described herein was three fold: to identify some of the mechanisms associated with beta cell dysfunction in feline diabetes mellitus, to develop techniques for the use of freshly isolated feline islets for the study islet cell pathogenesis in cats, and to study the mechanisms and utility of GLP-1 mimetics as a therapy of feline diabetes mellitus. Many aspects of the disease phenotype seen in cats are analogous to Type 2 diabetes mellitus in humans. Because of these similarities, much of our understanding of the mechanisms of disease pathogenesis and treatment of feline diabetes is extrapolated from work done in humans and in rodent models of human disease. Diabetes mellitus in both cats and people is a complex metabolic disease with no single cause or trigger. In both species the disease is associated peripheral insulin resistance, increased adiposity, inappropriate diet, and loss of beta cell mass. One of the most significant similarities is that cats are the only animals outside of

humans and non-human primates known to spontaneously produce pancreatic amyloid, making them particularly valuable as an animal model for human disease.

The most significant mechanisms associated with beta cell failure in people can be generally grouped into 5 categories: gluco-lipotoxicity, inflammation, oxidative stress, amyloid accumulation, and endoplasmic reticulum (ER) stress. To date, our assumptions regarding mechanisms of beta cell dysfunction in cats have largely been based on therapeutic outcomes, a hand-full of *in vivo* studies, and post-mortem examination. For example, the phenomenon of "diabetic remission" in cats is well described and occurs in cats most frequently after establishing aggressive glycemic control early after disease diagnosis. Moreover, in a single *in vivo* study, profound beta cell loss was described after a 10-day hyperglycemic clamp. These findings provide strong evidence that glucotoxicity is an important mediator of beta cell dysfunction. Studies describing the presence of inflammation, oxidative stress, or ER stress in cats are lacking. The association of amyloid accumulations with diabetes in cats as well as people is well described. There have not, however, been published studies testing the oligomeric amyloid hypothesis in cats.

Because of the gaps in our knowledge of whether oxidative stress and inflammation play a role in the development of feline diabetes, we first set out to characterize the markers of inflammation, oxidative stress, and amyloid in the pancreatic islets of normal and diabetic cats. In this study, we were the first to characterize the presence of inflammatory cytokine signaling (namely, IL-1β), and oxidative modifications (4-hydroxynonenal) within the islets of non-diabetic, apparently "healthy" cats. In contrast, the islets of diabetic cats were essentially absent or effaced with amyloid, making it difficult to draw conclusions about the presence (or absence) of inflammation of oxidative modification.

Identifying widespread staining for IL-1β and oxidative modification in the form of 4-HNE in healthy cats was unexpected. Because the islets in the diabetics were completely effaced and conclusions about islet inflammation or oxidative medication could not be made, we investigated the possibility that inflammation and oxidative modification are processes occurring earlier in disease development and will be absent by the time the islet is completely destroyed. Samples of pancreas from cats enrolled in another, unrelated, study were available from before and after 12 weeks of induced obesity and hyperglycemia (conditions typical of cats early in development of diabetes). The results from these paired samples suggest that obesity and hyperglycemia are associated with significant increases in inflammatory cytokine signaling and oxidative modification. Thioflavin-stained sections from these same cats failed to detect any islet amyloid deposits. These findings provides important clues to potential mechanisms of beta cell dysfunction not previously described in cats, namely the possible role of islet inflammation and oxidative stress and suggest that islet inflammation and oxidative stress may precede the development of amyloid. However, as these cats were experimentally induced diabetics, the disease process seen may not mirror naturally occurring disease.

Our next aim was to develop techniques for the isolation and culture of fresh feline pancreatic islets. Our ability to study the causes of beta cell dysfunction in cats is limited by the lack of physiologically appropriate, species-specific assays. Islet cell culture is commonly utilized in experiments studying the disease in humans and in rodent models of human disease. There is an array of various immortalized insulin producing cell lines available from rodent origin that can be very useful and primary islet culture is routinely utilized in pigs, people, and rodents. However, no such cell lines or islet isolation techniques exist for cats.

The result of this work was a technique for the isolation of highly pure pancreatic islets in sufficient numbers to be useful for a variety of laboratory assays. The islets that were isolated maintained adequate viability over a period of at least 5 days. Unfortunately, however, we were

unable to demonstrate reliable responsiveness to glucose stimulation. This was despite extensive modification of the culture system and use of several different culture media, culture substrates, and combinations of growth factors and antioxidants. The lack of reliable physiologic responsiveness does not, however, preclude the potential utility of freshly isolated pancreatic islets for assays evaluating other aspects of islet and beta cell physiology.

The origin of the inconsistent response of isolated feline islets is unclear. The "consistently inconsistent" nature of the islet function over many experiments over an extended period of time suggests that the reason for this failure lies in a treatment that all islets are exposed to: harvesting and transport conditions, isolation conditions, or culture conditions. As discussed in chapter 3, one possible reason stems from the methods of organ harvesting and transport. All cats were random-sourced and euthanized using the same protocol. Although there is no reason to expect interference with either the drugs used for sedation (ketamine, xylazine) or euthanasia (phenytoin-free pentobarbital overdose), it is possible that one of these drugs may significantly alter the metabolism of the islets and inhibit function. Cats euthanized using an alternative protocol would be required to test this hypothesis, but this was not possible given the restrictions on euthanasia protocols by the shelter staff. Cold ischemic time was limited to as short a period as possible, but the extended travel to get the tissues back to the laboratory meant at least one and a half hours cold ischemia time. It is possible that having the animals closer to the laboratory facility and potentially eliminating cold ischemia time may result in improved function.

Hand picking islets represented the single longest portion of the isolation procedure, during which time the islets were at ambient temperature exposed to room air with dithizone in the culture media. As an alternative to hand picking, density centrifugation must be re-visited. The small number of islets and apparently similar density of the islets to the exocrine tissue made our initial attempts at density centrifugation unsuccessful. But experimentation with different

compounds, speeds, and specific gravities may enhance the speed of recovery and limit the exposure of freshly isolated islets to harsh conditions.

A wide variety of culture media were tried during the optimization period prior to the experiments in chapter 3. The two culture media commonly utilized for islets in the literature are RPMI, which is commonly recommend for rodent islets, and CMRL, which is commonly recommended for human islets. The principle difference between these two media is the glucose concentration. The glucose concentration of the RPMI media is 10mmol/l and CMRL is 5.4mmol/l. As no difference was appreciated initially, CMRL was selected as the culture media because the lower glucose concentration left room to more easily manipulate experimental glucose concentrations and it was assumed that feline islets would behave more similarly to human islets than to rodent islets in response to glucose. Although fetal calf serum contains a number of growth factors, it is possible that feline islets require a specific combination that was not achieved in our experiments. Of specific interest are IGF-1 and 2, HGF, melanocortins, and glucagon. Glucagon has been shown to be a potent mediator of glucose stimulated insulin release in freshly isolated human islets. The concentration of glucagon in media was not determined in our experiments.

Collagenase tissue digestion can be very harsh to tissues and it is reasonable to expect collagenase digestion of the pancreas will alter islet morphology to some degree. As was discussed in chapter 3, altered islet composition morphology was associated with altered physiologic response to glucose in human islets. Morphology and cellular composition of our isolated islets was not assessed. Positive staining for dithizone was consistent with zinc-laden beta cells of islets, but integrity of islet basement membrane or altered islet composition secondary to digestion was not evaluated. Embedding of fresh islets for immunohistochemistry or electron microscopy is important to determining the composition of our isolated islets and any disruption in the islet connective tissue capsule. The techniques needed to effectively embed and

section small populations of islets were not mastered during our experiments, and collaboration with histologists with experience in this technique would be invaluable to resolving our technical problems.

Endoplasmic reticulum stress is a well-described mechanism believed to be associated with beta cell death in people and in rodent models. Studies using human and rodent islets and rodent cell cultures have demonstrated that GLP-1 mimetics are associated with improvement of beta cell function in the face of ER stress. Neither the role of ER stress nor the *in vitro* effects of GLP-1 mimetics have been evaluated in cats. Therefore, we designed experiments to first determine the effects of the ER stress inducing drugs thapsigargin and tunicamycin on freshly isolated pancreatic islets. Both drugs produced a predictable rise in caspase 3 activity, suggesting that ER stress can be chemically induced in our freshly isolated islets. We were then able to document an attenuation of caspase 3 activity in islets co-cultured with exenatide in addition to the ER stress-inducing drug. These results suggest that exenatide may have an islet cell sparing effect in freshly isolated feline islets exposed to ER stress. Although the data was suggestive of a protective effect of exenatide in islets treated with thapsigargin and tunicamycin, the small number of experiments makes it difficult to draw strong conclusions from the available data. Additional experiments under similar culture conditions are necessary to provide the power necessary to draw conclusions about whether exenatide protects pancreatic islets from both thapsigargin and tunicamycin. For reasons that may be related to our experiences in chapter 3 (isolation of islets), not all groups of islets isolated for this experiment showed an expected activation of caspase-3 in response to the ER stress-inducing drug. In fact, some control islets had relatively high base-line caspase-3 activation, suggesting activation of apoptotic pathways independent of the drugs. Additional work optimizing isolation of islets may also help solve this complication as well.

Our final aim was to evaluate the effectiveness of adjunctive therapy with exenatide in addition to basal insulin in newly diagnosed feline diabetics. Previous work completed by our group as well as by others suggested that exenatide was safe and as associated with a dose-dependent effect of augmenting serum insulin in response to glucose challenge. GLP-1 mimetics are routinely used in human Type 2 diabetics and have a proven record of safety and efficacy in improving quality of glycemic control in people. For this aim we recruited newly diagnosed, uncomplicated feline diabetics presented to the teaching hospital. A double-blind, placebo-controlled study design was used and cats were either treated with insulin alone or insulin with the addition of exenatide given twice daily. Recruitment proved to be a severely limiting factor, and the study was underpowered as a result. On the whole, there was no effect of exenatide detected in treated cats when exenatide was added to basal insulin. These results must be interpreted with caution, however, because of the small sample size.

At the time of diagnosis, most cats will have some volume of beta cell mass that can potentially be restored to function, a concept supported by the potential for remission of clinical disease in cats. Newly diagnosed cats, therefore, represent a heterogeneous population of cats with varying degrees of beta cell function superimposed on an already complicated genetic and metabolic backgrounds. In order to account for this wide ranging population, much larger studies will be required to provide adequate power to help identify which patients are more likely to benefit from incretin mimetic therapy. There are currently no tools available to quantify the actual beta cell reserve of a patient. Cats with little or no beta cell is unlikely to benefit significantly from exenatide therapy in terms of providing improved glycemic control as either a mono or adjunct therapy in a diabetic cat. However, in cats diagnosed early, cats in remission, or in “pre-diabetic” cats with impaired beta cell function, it is reasonable to expect a fairly large beta cell population remains and exenatide therapy may provide valuable support and improved outcomes by avoiding clinical signs of feline diabetes. Despite our limitations due to under-

powered data, it is reasonable to consider that that any difference in treatment outcomes in cats using this study design is likely to be small and clinically insignificant. Future studies with exenatide should take into consideration the importance of controlling for diet (a very important factor in the degree of post-prandial hyperglycemia), the value of longer-acting insulin (e.g. glargine), and the benefit of aggressive glycemic control immediately after diagnosis. Addressing each of these concerns is expected to improve treatment outcomes overall. Additionally, it may be unreasonable to expect a benefit of incretin therapy to be apparent after only 12 weeks of therapy without addressing the factors listed above. Perhaps overall improved glycemic control will be synergistic with incretin therapies and more significant long-term benefits can be achieved

The epidemic of feline diabetes is only expected to continue growing. Improved diets, more active lifestyles, and improved insulin formulations have improved our ability to provide long-term support and extended quality of life for diabetic cats. Our understanding of the root causes of the disease in cats is still developing. The availability of a technique for producing highly pure isolated feline islets provides a valuable tool for the study of beta cell dysfunction and the potential benefits of incretin therapy on beta cell function. The results of the work described here suggest that inflammation, oxidative stress, and misfolding IAPP all play a role in the development of beta cell dysfunction in the cat and that GLP-1 mimetic therapy may protect feline islets from ER stress which is known to play a role in beta cell loss in people. Our understanding of the best clinical application of GLP-1 mimetics such as exenatide is still developing and more robustly powered clinical trials are necessary to determine which subsets of diabetic cats may benefit from incretin therapy.

VITA

AARON MICHAEL HERNDON

Candidate for the Degree of

Doctor of Philosophy

Thesis: MECHANISMS OF BETA CELL DYSFUNCTION AND APPLICATION OF GLP-1 MIMETICS IN FELINE DIABETES MELLITUS

Major Field: Veterinary Biomedical Sciences

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Veterinary Biomedical Sciences at Oklahoma State University, Stillwater, Oklahoma in December, 2015.

Completed the requirements to earn Diplomat status in Small Animal Internal Medicine from the American College of Veterinary Internal Medicine in June, 2014.

Completed the requirements for a Doctor of Veterinary Medicine at Texas A&M University, College Station, Texas in May, 1998

Completed the requirements for a Bachelor of Science in Veterinary Science at Texas A&M University, College Station, Texas in May, 1995.

Experience:

Residency in Small Animal Internal Medicine and PhD in Veterinary Biomedical Sciences, Oklahoma State University, Stillwater, OK, 2010-2014
Small animal general practice veterinarian, Relief Veterinary Services, Keller, TX 2004-2010
Small animal general practice veterinarian, Parkside Animal Hospital, Keller, TX, 2006-2009
Small Animal emergency practice veterinarian, Veterinary Emergency Treatment Services, Oakdale, CT, 2003-2004
Small animal general practice veterinarian, Connecticut Veterinary Center, West Hartford, CT, 2002-2004
Small animal general practice veterinarian, Colchester Veterinary Hospital, Colchester, CT, 2006-2009
United States Army Veterinary Corps, New London Branch, Groton, CT, 1998-2001