PHARMACOKINETICS AND EFFECT OF PIOGLITAZONE IN OBESE, INSULIN-RESISTANT CATS

BY

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DISSERTATION

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Pioglitazone is a thiazolidinedione (TZD) insulin sensitizer approved for use in human type 2 diabetes mellitus. In addition to improving glycemic control, lipid profiles, and pancreatic \( \beta \)-cell function in diabetics, pioglitazone decreases hepatic triglyceride content in humans with non-alcoholic fatty liver disease. Cats, like humans, are predisposed by obesity to a form of diabetes characterized by peripheral insulin resistance and insulin secretory defects. They are also prone to a syndrome of severe hepatic triglyceride accumulation (hepatic lipidosis) during periods of decreased food intake. Therapeutic options for both diabetes and hepatic lipidosis in cats are limited, and additional treatment choices would be beneficial. The primary goal of this dissertation was to evaluate the effects of pioglitazone on insulin sensitivity in obese, insulin-resistant cats, to assess its potential for use in cats with diabetes mellitus. A secondary goal was to explore a technique for noninvasive measurement of liver fat in cats, to facilitate further study of pioglitazone or other agents in feline hepatic lipidosis. First, pharmacokinetic parameters for pioglitazone were determined in lean and obese cats, and data were used to design an oral dosing regimen for further study. Second, 1 and 3 mg/kg dosages of oral pioglitazone (Actos\textsuperscript{TM}) were administered to 12 obese, insulin-resistant cats in a placebo-controlled 3-way crossover design. Effects on insulin sensitivity were measured using intravenous glucose tolerance testing; additionally, indirect calorimetry was used to detect any species-specific effects of pioglitazone on substrate oxidation or energy expenditure. Third, \(^1\)H magnetic resonance spectroscopy (\(^1\)H MRS) was performed to determine hepatic fat content in the 12 obese cats and in 6 lean cats using a modification of established human methods, and fat content in liver samples from a separate group of 4 lean and 5 obese cats was also determined by chemical triglyceride assay. Oral pioglitazone was rapidly absorbed in cats, with a median bioavailability of 55\% and a
median half-life of 3 h. After 6 weeks of daily dosing, 3 mg/kg pioglitazone significantly increased insulin sensitivity, reduced insulin area under the curve during IVGTT, and lowered serum triglyceride and cholesterol concentrations in the obese cats. No changes in substrate oxidation or energy expenditure were observed. Pioglitazone concentrations after chronic dosing suggested dose-dependent absorption characteristics for this drug in cats. In the 12 obese and 6 lean cats, median liver fat percentages determined by $^1$H MRS (6.8% and 1.3% for the obese and lean cats, respectively), were similar to chemical assay values from the 5 obese and 4 lean cats (median liver fat 6% and 1.7%, respectively). Data obtained from these studies demonstrate a positive effect of 3 mg/kg pioglitazone on insulin sensitivity and lipid metabolism in obese cats, and suggest that further evaluation of the drug in cats with diabetes mellitus or other metabolic disorders is warranted. As well, they provide a foundation for continued development of $^1$H MRS techniques to quantify the effects of therapeutic interventions on liver fat in cats.
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CHAPTER I

Introduction

1.1 Introduction

Obesity is a significant problem in the domestic feline population (Scarlett et al., 1994; German, 2006). In both humans and cats, obesity is a risk factor for diabetes mellitus; obese cats are 2-4 times more likely to have the disease than are cats of normal body condition (Panciera et al., 1990; Scarlett & Donoghue, 1998). The mechanisms by which obesity predisposes to diabetes are unclear, but obese cats exhibit altered insulin secretion patterns and reduced peripheral insulin sensitivity (Biourge et al., 1997; Hoenig et al., 2000; Hoenig et al., 2007), and have subnormal expression of the insulin-sensitive glucose transporter GLUT4 (Brennan et al., 2004). In one study, insulin sensitivity in obese cats was estimated to decrease by 30% with each kilogram of excess body weight (Hoenig et al., 2007). Impaired peripheral insulin sensitivity is accompanied by compensatory hyperinsulinemia, which can eventually exhaust the secretory capacity of pancreatic β-cells and result in overt diabetes (Hoenig et al., 2000).

Feline obesity also predisposes to dyslipidemia (Jordan et al., 2008), and to abnormal fat deposition in the liver (hepatic lipidosis) following a period of rapid weight loss. Hepatic lipidosis can occur in non-obese as well as obese cats, and its causes are not well understood (Brown et al., 2007). However, this condition has some pathophysiologic similarities to human non-alcoholic fatty liver disease (NAFLD), a chronic hepatopathy associated with obesity, insulin resistance, and dyslipidemia (Holt et al., 2006; Ahmed & Byrne, 2009). Both feline
hepatic lipidosis and NAFLD are characterized by elevated concentrations of circulating non-esterified fatty acids (NEFAs) (Pazak et al., 1998; Holt et al., 2006; Brown et al., 2007). In hepatic lipidosis, these peripheral lipolytic products accumulate as triglyceride in hepatocytes, and can create life-threatening liver function deficits (Hall et al., 1997; Armstrong & Blanchard, 2009).

Current treatment options for feline diabetes with significant evidence of efficacy and safety include only insulin and the sulfonylureas. Insulin must be given by injection and carries the risk of hypoglycemia, both of which can be concerns for owners of diabetic cats (Niessen et al., 2010). Sulfonylureas increase insulin secretion by inhibiting an ATP-dependent potassium channel on β-cell membranes. Glipizide, a member of this drug class, has been used in diabetic cats, but long-term treatment with glipizide results in amyloid deposition in pancreatic islets (Hoenig et al., 2000). No pharmacological treatments are available for hepatic lipidosis; only aggressive nutritional support via a feeding tube has been shown to improve survival (Armstrong & Blanchard, 2009).

Thiazolidinediones (TZDs) are a class of oral antidiabetic drugs approved for human use. They are labeled for treatment of type 2 diabetes mellitus, and have recently shown therapeutic efficacy in patients with NAFLD (Belfort et al., 2006; Ahmed & Byrne, 2009). TZDs are agonists of peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear transcription factor that is a key regulator of adipogenesis (Lehrke & Lazar, 2005). The natural ligands for PPARγ are unknown, but the receptor possesses a low affinity for various endogenous fatty acids and eicosanoids (Walczak & Tontonoz, 2002). PPARγ is expressed abundantly in adipose tissue, and at low levels in other tissues, including pancreatic β-cells (Walczak & Tontonoz, 2002; Lehrke & Lazar, 2005).
In diabetic humans and rodents, the effects of TZD administration are an increase in whole-body insulin sensitivity, reduction of triglyceride content in non-adipose tissues such as liver and skeletal muscle, and lowering of serum triglyceride and/or NEFA concentrations (Lehrke & Lazar, 2005; Rasouli et al., 2005; Ravikumar et al., 2008; Bajaj et al., 2010). Although the molecular mechanisms for these changes have yet to be completely elucidated, activation of PPARγ is known to induce expression of numerous genes involved in glucose and lipid metabolism, including fatty acid binding and transport proteins, lipoprotein lipase (LPL), phosphoenolpyruvate carboxykinase (PEPCK), and GLUT4 (Sandouk et al., 1993; Walczak & Tontonoz, 2002). Mobilization of ectopic lipid may be linked to the adipogenic activities of PPARγ: agonist treatment increases the number of small, insulin-sensitive adipocytes in subcutaneous tissue, potentially shifting the balance for lipid uptake and storage away from liver and muscle (de Souza et al., 2001; Yki-Järvinen, 2004). Additionally, unlike sulfonylureas, TZDs appear to improve pancreatic β-cell function (Campbell et al., 2004; Gastaldelli et al., 2007; Wajchenberg, 2007). In human diabetics, this contributes to stable long-term glycemic control (Campbell et al., 2004; Wajchenberg, 2007).

Because TZDs are effective for treatment of human type 2 diabetes, they may be a promising therapeutic option for diabetes in cats. As well, because of their effects on lipid metabolism, they may be useful for treatment of feline hepatic lipidosis. Darglitazone, an experimental TZD, improved glucose and NEFA disposal during glucose tolerance tests in obese cats, while simultaneously reducing insulin concentrations (Hoenig & Ferguson, 2003). However, darglitazone did not complete clinical development, and of the approved TZDs (troglitazone, rosiglitazone, and pioglitazone), only pioglitazone has remained available for general prescription.
The primary goal of this dissertation was to describe the pharmacokinetics and effect of pioglitazone in obese, insulin-resistant cats, to assess its potential for use in cats with diabetes mellitus. A secondary goal was to explore a technique for noninvasive measurement of liver fat in cats, to facilitate further study of pioglitazone or other agents in feline hepatic lipidosis. Hypotheses were 1) that pioglitazone, appropriately dosed, would improve insulin sensitivity in obese cats, and 2) that $^1$H NMR spectroscopy could be successfully used to measure feline liver fat. Chapter 2 provides further explanation of the background and significance of this work. Chapters 3-5 address specific project aims, as described below. The last chapter of this dissertation (Chapter 6) summarizes project conclusions and outlines possible directions for future research.

**Specific Aim #1: Determine the pharmacokinetic properties of pioglitazone in cats.**

Single oral and intravenous doses of pioglitazone were administered in a crossover fashion to six healthy lean and six obese cats. Plasma drug concentrations were measured by high-performance liquid chromatography (HPLC), pharmacokinetic parameters were calculated using commercial software, and the data were used to select appropriate dosages for further study. This work is detailed in Chapter 3.

**Specific Aim #2: Evaluate the effects of pioglitazone on insulin sensitivity, glucose clearance, and non-esterified fatty acid (NEFA) disposal in obese cats.**

To test the hypothesis that pioglitazone would improve indices of insulin sensitivity in cats, 1 and 3 mg/kg dosages of pioglitazone were administered orally for 7 weeks to 12 obese cats, in a placebo-controlled 3-way crossover design. Changes in insulin sensitivity were assessed via
intravenous glucose tolerance testing, with measurement of glucose, insulin, NEFAs, and the adipokines adiponectin and leptin. Indirect calorimetry was also performed in order to detect any species-specific effects of pioglitazone on energy expenditure and substrate oxidation in cats. This work is detailed in Chapter 4.

**Specific Aim #3: Investigate the use of $^1$H MRS as a technique for measurement of hepatic triglyceride in cats.**

$^1$H magnetic resonance spectroscopy ($^1$H MRS) was performed in 6 lean and 12 obese cats using a modification of established human methods. To determine biological plausibility of the obtained values, chemical triglyceride assay of liver samples was performed in a separate group of 4 lean and 5 obese cats. This work is detailed in Chapter 5.
1.2 References


Chapter II

Background and Significance

2.1 Background and significance

Feline obesity and insulin resistance

At least 35% of domestic cats in the United States are overweight or obese (Scarlett et al., 1994; Lund et al., 2005), and a similar prevalence has been reported in Europe (Russell et al., 2000; Colliard et al., 2009). The relatively high occurrence of obesity in pet cats has been linked to sedentary, indoor lifestyles and consumption of calorically dense diets, factors similar to those implicated in the human “obesity epidemic” (Scarlett et al., 1994; Lund et al., 2005). Adipose tissue in mammals is now recognized as an endocrine organ rather than simply an inert storage depot, and accumulation of excess body fat has biochemical as well as mechanical consequences (Scherer, 2006). Specifically, obesity is associated with insulin resistance, or a subnormal response to insulin in target tissues, in both humans and cats (Buse et al., 2003; Hoenig, 2012).

a) Normal insulin secretion and action

In a healthy animal, insulin is secreted from pancreatic β-cells in a pulsatile fashion that reflects oscillations in plasma glucose concentration (Lefebvre et al., 1987; Buse et al., 2003). Glucose-stimulated insulin secretion is initiated when glucose diffuses into the β-cell through glucose transporter 2 (GLUT2); phosphorylation by pancreatic glucokinase traps the glucose intracellularly and allows it to enter the glycolytic pathway. ATP generation in glycolysis and/or oxidative phosphorylation causes blockade of ATP-dependent K⁺ channels on the β-cell
membrane, which promotes membrane depolarization and opening of voltage-gated calcium channels. The resulting calcium influx is the trigger for exocytosis of insulin-containing vesicles. When a sufficiently high dose of glucose is administered intravenously, two phases of insulin secretion are visible: a rapid first phase caused by the discharge of insulin from a specific pool of readily releasable granules, and a prolonged second phase that corresponds to the magnitude of the glucose stimulus (Buse et al., 2003; Seino et al., 2011).

Secreted insulin enters the circulation and binds to insulin receptors (IR), which are autophosphorylating tyrosine kinases located at the cell surface (Figure 2.1). After insulin binding, phosphorylation of multiple tyrosine residues on the intracellular subunits of the receptor allows recruitment and phosphorylation of scaffolding proteins, notably the insulin receptor substrates (IRS 1-4). Both phosphorylated IR and IRS interact with other proteins to initiate signaling cascades in metabolic and mitogenic pathways (Saltiel & Kahn, 2001). Downstream effectors of insulin signaling may be protein kinases and phosphatases, transcription factors, or proteins involved in vesicle trafficking (Saltiel & Kahn, 2001; Krüger, 2008); the protein kinases phosphatidylinositol-3-kinase (PI(3)K) and Akt are key mediators of the effects of insulin in metabolic pathways, whereas PI(3)K and mitogen-activated protein kinases (MAP kinases) are important for its growth-promoting effects (Saltiel & Kahn, 2001).

In many tissues, including muscle and fat, an acute effect of insulin stimulation is translocation of vesicles containing the glucose transporter GLUT-4 to the plasma membrane. This occurs through insulin receptor (IR)- and IRS-1-induced activation of small GTPases, and produces a dramatic increase in glucose uptake within seconds of insulin binding (Guyton & Hall, 2000; Leto & Saltiel, 2012). The majority (at least 75%) of whole-body insulin-stimulated glucose uptake is mediated by skeletal muscle (Saltiel & Kahn, 2001; Leto & Saltiel, 2012). IR
activation in muscle also causes rapid phosphorylation of the enzyme glycogen synthase kinase, reducing its inhibition of glycogen synthase and increasing storage of the entering glucose as glycogen. In adipocytes, entering glucose forms alpha-glycerol phosphate, a precursor for triglyceride synthesis. Insulin increases lipid uptake and storage in these cells by activating endothelial lipoprotein lipase (LPL), fatty acid transport proteins, and lipid synthetic enzymes. Additionally, activation of a cAMP-specific phosphodiesterase by insulin signaling in adipocytes inhibits the enzyme hormone-sensitive lipase, profoundly suppressing peripheral lipolysis and decreasing release of non-esterified free fatty acids (NEFAs) into circulation (Guyton & Hall, 2000).

In the liver, insulin signaling through IRS-2 increases the expression and activity of glucokinase and glycogen synthase, which cause glucose phosphorylation and glycogen storage, respectively. When hepatic glycogen stores are replete, excess glucose is metabolized by glycolysis, and insulin-mediated activation of the enzyme acetyl CoA carboxylase promotes the synthesis of triglycerides. Ultimately, these triglycerides may be secreted as very low density lipoproteins (VLDL) (Guyton & Hall, 2000); however, VLDL production is acutely inhibited by insulin in the postprandial state (Gibbons et al., 2004). Insulin signaling also inhibits hepatic glycogenolytic enzymes (glycogen phosphorylase and glucose-6-phosphatase), reduces the quantity and activity of enzymes required for gluconeogenesis (through antagonism of the transcription factor FOXO1 by Akt), and decreases the availability of gluconeogenic substrates by impeding protein catabolism in other tissues (Guyton & Hall, 2000; Kahl & Roden, 2012). These changes contribute to insulin-dependent suppression of hepatic glucose production.
b) Alterations in obesity

In humans, obesity produces insulin resistance in all three major metabolic organs (skeletal muscle, adipose tissue, and the liver). This has been demonstrated through the use of a multiple-dose euglycemic-hyperinsulinemic clamp (EHC) with isotopic tracers, which compared rates of peripheral glucose uptake, fatty acid (palmitate) appearance, and hepatic glucose production between lean and obese individuals at low and high physiologic insulin concentrations. During the low-dose insulin infusions, glucose and palmitate rate of appearance ($R_a$, expressed per kilogram of fat-free mass) were not appropriately suppressed by insulin in obese subjects, and insulin-stimulated glucose uptake was decreased at all insulin infusion rates. Also, at all insulin infusion rates, systemic plasma insulin concentrations were 20-50% greater in obese than in lean subjects (Conte et al., 2012). The latter finding reflects the fact that ß-cells respond to insulin resistance with a compensatory increase in insulin secretion (Kahn et al., 1993; Buse et al., 2003).

Although a multiple-dose EHC has not been reported in cats, reduced glucose clearance during intravenous glucose tolerance tests (IVGTT) has been documented in obese cats by multiple investigators (Biourge et al. 1997; Wilkins et al., 2004)). As mentioned previously, one study using a single-dose EHC found that insulin sensitivity decreased by 30% for each 1-kg increase in body weight (Hoenig et al., 2007). Obese cats also exhibit basal hyperinsulinemia (Jordan et al., 2008; Hoenig et al., 2011) and exaggerated second-phase insulin secretion during IVGTT (Wilkins et al., 2004; Kley et al., 2008). Additionally, they are dyslipidemic, with increased concentrations of triglyceride-rich VLDL and small dense LDL (Jordan et al., 2008). These lipoprotein changes occur when insulin fails to suppress VLDL secretion, and similar
abnormalities are seen in insulin-resistant humans and rodents (Ginsberg et al., 2005; Sørenson et al., 2011).

The mechanisms by which obesity leads to insulin resistance are not well understood. However, abnormal fatty acid trafficking in human obesity appears to result in increased delivery of lipids to lean tissue, and in intracellular accumulation of lipid intermediates such as long chain fatty acyl CoAs (LC-FA CoAs), diacylglycerol (DAG), and ceramides (Samuel et al., 2009; McQuaid et al., 2011). These intermediates are thought to alter insulin signaling through phosphorylation of serine residues on the insulin receptor (IR) or its substrates (Muioio & Newgard, 2008). The link between lipid metabolites and serine phosphorylation may occur through isoforms of the protein kinase C (PKC) family (PKCθ in muscle, and PKCε in the liver), which are activated by DAG and other lipid derivatives (Peterson & Shulman, 2006; Muoio & Newgard, 2008; Kahl & Roden, 2012), or through activation of other serine kinases such as c-Jun N-terminal kinase (JNK) (Muioio & Newgard, 2008). Potential triggers for the latter include metabolic overload in the endoplasmic reticulum (ER), and exposure to inflammatory cytokines such as TNF-α and IL-6 (Muioio & Newgard, 2008).

Adipose tissue in obese humans and rodents is infiltrated by macrophages with a pro-inflammatory phenotype (Wellen & Hotamsligil, 2005; Glass & Olefsky, 2012). TNF-α and IL-6 may be released either from these macrophages or from dysfunctional adipocytes, resulting in local and systemic elevations of inflammatory markers (Park et al., 2005; Glass & Olefsky, 2012). In contrast to the situation in humans, obesity-induced macrophage infiltration has not been reported in cats, and obese cats do not have elevated plasma concentrations of TNF-α or IL-6 (Hoenig et al., 2013). However, obese cats do have increased TNF-α mRNA in adipose tissue (Hoenig et al., 2006), suggesting inflammatory mediators may still participate in feline insulin
resistance by acting locally (Hoenig et al., 2013). Obese cats are also likely to have abnormal fatty acid trafficking, as they have increased LPL expression in muscle, decreased LPL expression in adipose tissue, and elevated intramyocellular and extramyocellular lipid concentrations (Wilkins et al., 2004; Hoenig et al., 2006).

Despite the presence of multi-organ insulin resistance in obesity, the basal rate of adipose tissue lipolysis is actually lower in obese than in lean humans (Mittendorfer et al., 2009), although total NEFA $R_a$ and serum NEFA concentration may be greater because of increased fat mass (Karpe et al., 2009; Mittendorfer et al., 2009). As well, a recent study of human VLDL kinetics found a lower rate of hepatic glucose production (per kilogram of fat-free mass) in obese than in lean men with normal glucose tolerance (Sørenson et al., 2011). This is in harmony with other studies which have reported no increase in the basal rate of hepatic glucose production in otherwise healthy obese subjects (Gastaldelli et al., 2004). Together, these findings illustrate that 1) insulin resistance is manifest in a tissue-specific manner, consistent with different insulin dose-response relationships for different metabolic processes (Yki-Järvinen et al., 1987), and 2) under physiologic conditions, certain consequences of insulin resistance can be masked by compensatory hyperinsulinemia (Conte et al., 2012).

Similar to the human situation, serum NEFA concentrations in obese cats are frequently, but not always, elevated (Wilkins et al., 2004; Jordan et al., 2008), and both fasting and postprandial hepatic glucose production are lower in obese cats than in lean cats when normalized for body weight (Kley et al., 2009; Hoenig et al., 2011). As a result of their ability to maintain control of hepatic glucose production, obese cats have glucose concentrations similar to those of lean cats when monitored throughout the day (Hoenig et al., 2012). Nonetheless, the underlying insulin resistance of obese cats is likely to contribute to their susceptibility to
metabolic disorders, including diabetes mellitus (Scarlett & Donoghue, 1998). One potential explanation for the connection between obesity-induced insulin resistance and diabetes is that the ER stress, oxidative damage, and inflammatory changes resulting from fuel surfeit interfere with insulin signaling in β-cells, as they do in other tissues (Prentki & Nolan, 2006). Since insulin signaling is necessary for β-cell growth and survival, such interference could ultimately promote β-cell loss (Rhodes, 2005; Kasuga, 2006). Another possible explanation involves the increased secretory demand associated with insulin resistance, which has been postulated to lead to depletion of pancreatic insulin stores and β-cell exhaustion (Prentki & Nolan, 2006). However, the latter mechanism has been demonstrated primarily in situations of pre-existing β-cell compromise (e.g. Matsuda, et al., 2002), and many obese cats and humans never develop diabetes, despite years of insulin resistance. Therefore, apart from playing a direct role in β-cell dysfunction, insulin resistance may simply increase the likelihood of metabolic decompensation when other genetic or environmental stresses on the β-cell are present.

*Feline diabetes mellitus*

Diabetes mellitus (DM) affects 0.4-1% of domestic cats, and is more common in those that are male, neutered, aged, or inactive, in addition to those that are obese (Prahl et al., 2007; McCann et al., 2007). In humans, DM is classified as type 1 or type 2 based on etiopathogenesis; type 1 is associated with absolute insulin deficiency and immune-mediated β-cell destruction, whereas type 2 is characterized by a combination of insulin resistance and potentially reversible β-cell dysfunction. The most frequently encountered form of DM in cats resembles human type 2 DM in several respects (Henson & O’Brien, 2006; Hoenig, 2012). First, although diabetic cats have a 50% loss of β-cell mass (O’Brien et al., 1986), it is not immune-
mediated, as β-cell antibodies have not been identified in untreated diabetic cats (Hoenig et al., 2000b). Second, the emergence of feline DM depends on the balance between insulin resistance and β-cell function; induction of insulin resistance by corticosteroids, progestagens, or weight gain can precipitate clinical disease in susceptible individuals (Hoenig et al., 2000a; Sieber-Ruckstuhl, 2008; Reusch, 2010). Third, both human type 2 and feline DM are typified histologically by deposition of amyloid in pancreatic islets (O’Brien et al., 1986; Hoenig et al., 2000a; O’Brien, 2002).

Islet amyloid is found in 90% of human type 2 diabetics and in 65-90% of diabetic cats (Johnson et al., 1986; O’Brien, 2002). It is derived from amylin, also called islet amyloid polypeptide (IAPP) (Westermark et al., 1987); this polypeptide is co-secreted with insulin, and, consequently, is hyper-secreted in insulin-resistant states (Henson et al., 2011). In cats, humans, and certain non-human primates that develop type 2 DM (e.g. macaques, baboons), the midportion of amylin has an amino acid sequence that promotes the formation of secondary structure, including β-pleated sheets and amyloid fibrils (Betsholtz et al., 1989; O’Brien, 2002; Guardado-Mendoza et al., 2009). These amyloid aggregates are cytotoxic in vitro, as incubation of islets with amyloid fibrils, or expression of human amylin in cultured cells, results in apoptosis (O’Brien, 2002).

In a study evaluating pancreatic lesions in 6 diabetic cats, 6 non-diabetic cats with impaired glucose tolerance (IGT), and 6 young non-diabetic cats with normal glucose tolerance (NGT), islet amyloid was absent in all cats with NGT, but was present in all diabetic cats and in 2/6 nondiabetic cats with IGT (O’Brien et al., 1986). In the diabetic cats and in the 2 IGT cats with amyloid deposits, β-cell mass was decreased, suggesting a relationship between amyloid deposition and β-cell apoptosis in vivo as well as in vitro (O’Brien et al., 1986). Pancreatic
Amyloid deposits are also present in approximately 45% of older non-diabetic cats, although they are not as extensive as in diabetic cats (Yano et al., 1981). Therefore, amyloid deposition is not the sole determinant of β-cell failure in feline DM; however, it may contribute by reducing β-cell functional reserve (O’Brien, 2002; Henson & O’Brien, 2006).

As discussed above, superimposition of insulin resistance on defective β-cell function, or vice versa, is the likely trigger for clinical DM. One study has examined the progression to diabetes in partially pancreatectomized cats rendered insulin-resistant by administration of growth hormone and dexamethasone. An early sign of developing DM in these cats was loss of first-phase insulin secretion; this was followed by a reduction in total insulin secretion and by development of abnormal glucose clearance. Eventually, a further decline in insulin secretion allowed the onset of fasting hyperglycemia (Hoenig et al., 2000a). The pattern of insulin secretory dysfunction during the onset of human type 2 DM is similar (Weyer et al., 1999), and in humans, the final increase in glucose concentration is due to a rise in endogenous glucose production (Weyer et al., 1999).

Hyperglycemia in diabetic cats may perpetuate or worsen impaired β-cell function. The detrimental effects of hyperglycemia on β-cell mass and function are referred to as glucotoxicity, and have been demonstrated in multiple species both in vitro and in vivo (Poitout & Robertson, 2008). In one study, chronic exposure of cultured β-cells to high glucose concentrations reduced binding of the transcription factors MafA and PDX-1 to the insulin promoter, inhibiting insulin production and secretion. These effects were reversible when the cells were transferred to a low-glucose medium (Poitout & Roberton, 2008). A similar loss of insulin secretion occurs with hyperglycemia in numerous rodent models (Leahy et al., 1992), and in healthy cats, clamping of blood glucose at 30 mmol/L (540 mg/dL) for 10 days caused loss of glucose-stimulated insulin
secretion, depletion of pancreatic insulin stores, and ß-cell apoptosis (Zini et al., 2009). Free fatty acids also impair insulin secretion, reduce insulin gene expression, and induce ß-cell death in vitro (lipotoxicity) (Poitout & Roberston, 2008). In vivo, however, these effects are manifest only in the presence of high glucose concentrations (Poitout & Robertson, 2008).

At the time of diagnosis, many diabetic cats have low to low-normal insulin concentrations, indicating substantial ß-cell functional deficits (Crenshaw & Peterson, 1996). However, recovery of ß-cell function and clinical remission can occur in some cats with appropriate treatment (Nelson et al., 1999; Zini et al., 2010). Clinical remission is defined as maintenance of normoglycemia and lack of clinical signs of DM for at least one month after cessation of therapy (Zini et al., 2010). In one study of 10 cats with clinical remission, 6 cats had undetectable insulin concentrations prior to treatment, and all lacked an insulin secretory response to intravenous glucagon. After treatment with either insulin or glipizide (an insulin secretogogue), all 10 cats exhibited a normal response to glucagon stimulation (Nelson et al., 1999). Recovery of insulin secretion with reversion to a non-insulin-dependent state has also been described in humans treated for type 2 DM, either with insulin or with oral hypoglycemic agents (Weng et al., 2008). This phenomenon is thought to result, at least in part, from alleviation of glucotoxicity (Weng et al., 2008).

Diabetic remission in cats does not imply an absence of ß-cell pathology. In the study by Nelson et al, 5/5 cats from which pancreatic biopsies were obtained had histologic abnormalities (consisting of decreased islet numbers, islet amyloidosis, vacuolar degeneration, and swollen ß-cells) (Nelson et al., 1999). Other studies have found that cats with diabetic remission are susceptible to relapse; in a retrospective examination of 275 diabetic cats admitted to a referral hospital, of which 50% achieved clinical remission, median remission duration was 114 days.
Therefore, cats that have been diabetic at any point in the past should always be considered at risk for future disease.

Clinical signs associated with DM in cats are similar to those observed in other species, including polyuria, polydipsia, lethargy, and weight loss. Appetite may be increased or decreased depending on the stage of disease (Crenshaw & Peterson, 1996). On laboratory evaluation, hypercholesterolemia, hypertriglyceridemia, hyperbilirubinemia, and elevated liver enzymes are commonly present in addition to hyperglycemia and glycosuria (Crenshaw & Peterson, 1996); elevated liver enzymes and bilirubin are presumed to be due to hepatic lipid accumulation, which was identified at necropsy in 35% of diabetic cats in one study (Goossens et al., 1998). Other possible complications of feline DM are polyneuropathy, manifest as a plantigrade stance, and diabetic ketoacidosis (DKA). DKA develops when absolute or relative insulin deficiency co-exists with an excess of counterregulatory hormones (Schade & Eaton, 1979).

Currently, the mainstay of treatment for feline DM is exogenous insulin, in conjunction with dietary management. Insulin treatment (typically with protamine zinc (PZI), glargine, or lente preparations) can achieve clinical control of DM in 60-85% of cats within 2-4 months (Michiels et al., 2008; Weaver et al., 2006; Nelson et al., 2009), and remission rates of >50% have been reported when insulin is combined with a high-protein, low-carbohydrate diet (Bennett et al, 2006). Potential drawbacks of insulin therapy include hypoglycemia, which occurs in 41-64% of insulin-treated cats and is symptomatic in up to 20% (Michiels et al., 2008; Nelson et al., 2009), and day-to-day variation in efficacy due to difficulties associated with product stability or injection technique (Reusch, 2010). The need to give insulin by injection and
the possibility of hypoglycemia were both identified as concerns in a recent survey of diabetic cat owners (Niessen et al., 2010).

Glipizide, as mentioned above, has also been used clinically in diabetic cats. This drug is a member of the sulfonylurea class of insulin secretagogues, which act by binding to and inhibiting a $K^+$ channel on $\beta$-cell membranes. Unfortunately, glipizide is effective in only 30-40% of diabetic cats (Feldman et al., 1997), and long-term treatment with glipizide leads to amyloid deposition in feline pancreatic islets (Hoenig et al., 2000a). Hypoglycemia and hepatotoxicity are also potential adverse effects of glipizide treatment (Feldman et al., 1997). Given these limitations, other options for treatment of feline DM, especially for owners who are unwilling or unable to attempt insulin therapy, would be beneficial. A selection of other oral agents (acarbose, metformin) has been investigated in diabetic cats, but with little success (Mazzaferro et al., 2003; Nelson et al., 2004).

**Feline hepatic lipidosis**

Hepatic lipidosis (HL) is the most commonly diagnosed liver disease of cats in North America (Armstrong & Blanchard, 2009). It is characterized by marked hepatocellular triglyceride accumulation (averaging 34% of liver weight in affected cats) and usually develops after a period of partial or complete anorexia (Hall et al., 1997; Center et al., 1993; Armstrong & Blanchard, 2009). Anorexia and HL can occur as a result of stress, diet changes, or accidental food restriction in otherwise healthy cats (designated primary HL), or can be related to the presence of concurrent disease (so-called secondary HL) (Armstrong & Blanchard, 2009). No breed or sex predisposition for feline HL is recognized, but, like feline DM, the disease is more common in obese cats than in cats of normal body condition (Center et al., 1993).
The pathogenesis of feline HL is thought to involve 1) a negative energy balance, 2) induction of hormonal alterations that promote excessive peripheral lipolysis, and 3) delivery of high levels of endogenous lipids to the liver (Armstrong & Blanchard, 2009). In a study comparing hormone and lipid concentrations in cats with HL, anorexic cats with other liver disease (cholangiohepatitis), and healthy control cats, cats with HL had hypoinsulinemia, a decreased insulin:glucagon ratio, and a threefold elevation of serum NEFAs relative to control cats (Brown et al., 2000). Interestingly, mean serum NEFA concentration in HL cats was almost twice that of similarly anorexic cats with cholangiohepatitis, although this may have been a result of small sample size in the cholangiohepatitis group, or of difference in body condition between the groups (Brown et al., 2000). NEFAs derived from peripheral lipolysis are the source of liver triglyceride in cats with HL; this is confirmed by the fact that liver lipid composition in HL cats, but not in control cats, corresponds closely with that of peripheral adipose depots (Hall et al., 1997).

Peripheral lipolytic products delivered to the liver may be disposed of in one of two ways: transport into mitochondria for β-oxidation, or re-esterification and storage in cytosolic droplets (McGarry & Foster, 1980). A proportion of stored triglyceride is later secreted as VLDL (Gibbons et al., 2004). β-oxidation would be expected to be particularly active in the fasting state, as production of malonyl CoA, the primary inhibitor of fatty acid transport into mitochondria, is diminished in the absence of insulin (McGarry et al., 1989). Indeed, cats with HL have elevated concentrations of the ketone β-hydroxybutyrate, an indirect product of β-oxidation (Pazak et al., 1998). Also, they have increased concentrations of VLDL, which are ascribed to an increase in VLDL secretion (Pazak et al., 1998). Clearly, however, neither β-
oxidation nor VLDL secretion in HL cats occurs at a rate sufficient to prevent triglyceride accumulation.

Some investigators have suggested that this is because of compartmentalization of triglyceride pools in the hepatocyte: triglyceride in vacuoles is destined for VLDL secretion, and whereas rates of both β-oxidation and VLDL secretion are finite, the capacity for triglyceride storage is not (Pazak et al., 1998). Thus, excess NEFA delivery may promote excess triglyceride storage, which persists despite maximal β-oxidation and VLDL secretion (Pazak et al., 1998). Others have focused on defects in mitochondrial or peroxisomal oxidation as the cause of failure to mobilize hepatic triglyceride (Ibrahim et al., 2003). Carnitine is an essential component of carnitine palmitoyltransferase, the major mitochondrial fatty acid transporter; consequently, supplementation of this nutrient during fasting in cats has been investigated (Ibrahim et al., 2003). L-carnitine supplementation increased fatty acid oxidation during weight loss in obese cats (Ibrahim et al., 2003), but did not attenuate liver lipid accumulation or prevent the development of experimentally induced HL (Blanchard et al., 2002; Ibrahim et al., 2003).

Among other supplements that have been examined for prevention of HL, only high dietary protein has been shown to have a beneficial effect (Biourge et al., 1994). In obese cats undergoing 4 weeks of fasting, hepatic lipid accumulation still occurred in those fed a high-protein diet, but to a lesser degree than in those fed high-carbohydrate, high-fat, or control diets (Biourge et al., 1994). A syndrome very similar to feline HL occurs in other small carnivores, including mink and European polecats (Bjornvad et al., 2004; Nieminen et al., 2009). Therefore, the susceptibility of cats to HL may well be related to their unique metabolic needs and carnivorous status, although a deficiency of any particular nutrient has yet to be identified.
In cats with HL, excess hepatic triglyceride interferes with liver function and bile flow, producing clinical changes such as icterus, hyperbilirubinemia, elevated liver enzymes, and hypoalbuminemia (Center et al., 1993). Vitamin-K responsive coagulopathy, presumed to be due to lack of absorption of fat-soluble vitamins, and electrolyte abnormalities may also occur (Armstrong & Blanchard, 2009). The institution of aggressive nutritional support via nasoesophageal, esophagostomy, or gastrostomy tubes has improved the survival rate of cats with HL from close to 0% to 60-90% (Jacobs et al., 1989; Armstrong & Blanchard, 2009). However, these measures may not be feasible for some cat owners, and, as for feline DM, additional treatment options would be desirable.

Thiazolidinediones

Thiazolidinediones (TZDs, or “glitazones”) are orally administered insulin sensitizers marketed for use in human type 2 diabetes. The antihyperglycemic activity of this class of compounds was discovered empirically in the late 1970s by Takeda Chemical Industries, during an investigation of lipid-lowering compounds in diabetic rodent models (Sohda et al. 1982; Grossman & Lessem, 1997). In diabetic rats and mice, TZDs reduced elevated plasma glucose, insulin, and triglyceride concentrations, improved insulin-stimulated peripheral glucose uptake, and reduced hepatic glucose production, without increasing insulin secretion or causing hypoglycemia (Ikeda et al., 1990; Sugiyama et al., 1990; Grossman & Lessem, 1997; Day, 1999). They also improved glucose tolerance and mitigated hyperlipidemia and hyperinsulinemia in nondiabetic obese rodents (Ikeda et al., 1990; Grossman & Lessem, 1997; Day, 1999). In lean or streptozotocin-induced diabetic animals, however, TZDs did not alter glucose tolerance or plasma glucose concentration (Ikeda et al., 1990; Day et al, 1999). These
results suggested that the TZDs produced their effects by enhancing the action of insulin in insulin-resistant states (Ikeda et al., 1990; Hofmann & Colca, 1992; Grossman & Lessem, 1997).

In the 1990s, three TZDs (troglitazone, rosiglitazone, and pioglitazone) were approved for treatment of human type 2 DM (Yki-Järvinen, 2004). Troglitazone was withdrawn from the US market in 2000 (three years after product launch) because of a low incidence of idiosyncratic liver toxicity, but rosiglitazone and pioglitazone became widely prescribed during the subsequent decade (Yki-Järvinen, 2004; Alexander et al., 2008). In diabetic humans, TZDs improved glycemic control by increasing peripheral insulin sensitivity and reducing endogenous glucose production, similar to their effects in animal models (Aronoff et al., 2000; Yamasaki et al., 1997; Miyazaki et al., 2004; Yki-Järvinen, 2004). They also decreased fasting and/or postprandial NEFA concentrations and augmented insulin-mediated suppression of adipose tissue lipolysis (Mayerson et al., 2002; Goldberg et al., 2005; Tan et al., 2005; Gastaldelli et al, 2009). The effects of TZDs on lipoproteins have been shown to vary depending on the specific agent: whereas pioglitazone decreases triglycerides, increases HDL cholesterol, lowers LDL particle number, and increases LDL particle size in human diabetics, rosiglitazone has opposite effects on triglycerides and LDL particle number, and increases HDL and LDL particle size and HDL particle number to a lesser degree (Goldberg et al., 2005).

In addition to their benefits in terms of glycemic control, TZDs improve indices of β-cell function in human diabetes (Miyazaki et al., 2002; Ovalle & Bell, 2004; Gasteldelli et al., 2007; Kutoh & Fukushima, 2009). Rosiglitazone has been demonstrated to restore first-phase insulin secretion during IVGTT, and both pioglitazone and rosiglitazone increase the disposition index (a measure of glucose-stimulated insulin secretion relative to insulin resistance) in diabetic patients (Gastaldelli et al., 2007). These data have been supplemented by an investigation in
rodent models, which showed that diabetes-induced changes in pancreatic islet structure, including depletion of secretory granules and infiltration of exocrine cells, were prevented by TZD treatment (Diani et al., 2004). Clinically, the consequences of β-cell functional improvement and preservation by the TZDs are durable long-term glycemic control (Campbell et al., 2004; DeFronzo, 2010) and reduction of diabetes risk in susceptible populations (Xiang et al., 2006; DeFronzo et al., 2011). In a large cohort of humans with impaired glucose tolerance ("prediabetics"), pioglitazone reduced the risk of conversion to diabetes by 72% (DeFronzo et al., 2011).

TZDs were approved only for treatment of type 2 DM. However, they have been investigated in several other insulin-resistant conditions, including lipodystrophies, polycystic ovary syndrome, and non-alcoholic fatty liver disease (NAFLD) (Yki-Järvinen, 2004). The latter is a term for a spectrum of conditions characterized by hepatic triglyceride accumulation, ranging from simple steatosis to severe steatohepatitis and fibrosis (Ahmed & Byrne, 2009). Human NAFLD differs from feline hepatic lipidosis in that it typically develops in the context of overnutrition and hyperinsulinemia; however, it is similar in that it involves high circulating endogenous NEFAs, which are the source of stored triglyceride in hepatocytes (Byrne, 2012). In both type 2 diabetic patients and in nondiabetic patients with NAFLD, TZDs reduce liver triglyceride by 30-50% (Belfort et al., 2006; Ravikumar et al, 2008; Yki-Järvinen, 2009). Pioglitazone also lowers intracellular triglyceride and long chain fatty acyl-CoA in muscle of diabetic subjects (Ravikumar et al., 2008; Bajaj et al., 2010).
Mechanism of action

Although their mechanism of action was unknown at the time of discovery, it has since become clear that TZDs are agonists of the peroxisome proliferator activated receptor gamma (PPARγ) (Lehmann et al., 1995; Tontonoz & Spiegelman, 2008). The PPARs (PPARα, PPARβ/δ, and PPARγ) are transcription factors belonging to the nuclear hormone receptor superfamily; they have a wide tissue distribution and play key regulatory roles in metabolism, cellular differentiation, and cell growth (Menendez-Gutierrez, et al., 2012). PPARγ, in particular, is crucial for adipocyte development, lipid transport and storage, and whole-body glucose homeostasis (Menendez-Gutierrez, et al., 2012). Of the two protein isoforms of PPARγ that have been described, PPARγ1 is expressed at low levels in many tissues (most abundantly in colon, macrophages, and vascular endothelium), whereas PPARγ2 is highly expressed in adipocytes (Vidal-Puig et al., 1997; Zieleniak et al., 2008).

Like other nuclear receptors that are heterodimeric with the retinoid X receptor (RXR) (e.g. thyroid hormone receptor), PPARγ is bound to co-repressors in the nucleus in its unliganded state. Association with a PPARγ agonist stabilizes a receptor conformation that promotes dissociation of co-repressors, recruitment of co-activators, heterodimerization with RXRα, and binding of the complex to hormone response elements in DNA (Rosen & Spiegelman, 2001). Genome-wide chromatin immunoprecipitation studies in adipocytes and macrophages have revealed thousands of functional binding sites for the PPARγ-RXRα heterodimer (Lefterova et al., 2010). PPAR response elements (PPREs) tend to be in enhancer regions far from transcription start sites, analogous to the situation for glucocorticoid and other hormone receptors. Many are adjacent to binding sites for CCAAT-enhancer binding protein α (C/EBPα), a transcription factor that participates in a self-reinforcing loop to perpetuate PPARγ
expression (Nielsen et al., 2008; Siersbæk et al., 2010). The accessibility of PPREs to the PPARγ–RXRα complex is regulated by patterns of histone methylation that vary by cell type, allowing a tissue-specific gene expression profile (Lefterova et al., 2010; Siersbæk et al., 2010).

In adipocytes and adipocyte precursors, PPREs are present in the majority of genes controlling glucose and lipid metabolism, including those coding for aP2/FABP4 (an adipocyte-specific fatty acid binding protein), lipoprotein lipase (LPL), phosphoenolpyruvate carboxykinase (PEPCK), and acyl CoA synthetase (ACS) (Henke, 2004; Siersbæk et al., 2010; Nielsen et al. 2008). Activation of PPARγ in preadipocytes or cultured 3T3 fibroblasts is sufficient to drive terminal adipocyte differentiation, characterized phenotypically by lipid accumulation and acquisition of insulin sensitivity (Wu et al., 1999; Tontonoz et al., 1994; Rosen & MacDougald, 2006). In brown adipocytes, additional features of terminal differentiation are PPARγ-mediated increases in mitochondrial biogenesis and expression of the uncoupling protein 1 (UCP1), a proton channel that allows dissipation of energy derived from oxidative phosphorylation as heat (Tai et al., 1996). Ablation of PPARγ in mature white and brown adipocytes results in the loss of their typical phenotypes, disordered lipid storage, and necrosis within a few days (Wu et al., 1999; Imai et al., 2004), demonstrating that PPARγ is necessary for adipocyte survival and function as well as for adipogenesis. PPARγ also controls expression of genes involved in lipid uptake and transport in other tissues, promotes cellular differentiation in macrophages, epithelium and placenta, and suppresses secretion of inflammatory molecules in both macrophages and adipocytes (Barak et al., 1999; Rosen & Spiegelman, 2001).

A high-affinity natural ligand for PPARγ has not been identified. However, the receptor possesses a low affinity for various endogenous fatty acids and eicosanoids (Walczak & Tontonoz, 2002), and has a relatively large hydrophobic ligand binding pocket that can
accommodate multiple ligands simultaneously (Liberato et al., 2012). These characteristics have led to speculation that PPARγ functions as a “lipid sensor” that regulates metabolic processes in response to endogenous lipid cues (Henke, 2004; Lehrke & Lazar, 2005). Structurally, the TZDs resemble fatty acids in some respects, having an acidophilic “head” group, a short spacer region, and a hydrophobic “tail.” The TZD “head” group forms hydrogen bonds with specific residues in the ligand-binding pocket of the receptor, fixing the position of the C-terminal activation function helix (AF2) and augmenting its ability to bind coactivators. Variations in the cyclic “tail” among molecules in the TZD class confer different receptor affinities, transactivation potential, and clinical properties (Henke, 2004; Loiodice & Pochetti, 2011). In addition to classical agonism of PPARγ, the presence of a TZD in the ligand-binding pocket may prevent post-translational receptor modifications that would suppress transcriptional activity (Floyd & Stephens, 2012); this has been demonstrated for rosiglitazone and the serine kinase Cdk5 (Choi et al., 2010).

For the TZDs, adipose tissue is likely to be a major site of action through direct PPARγ activation. PPARγ is abundant in mature adipocytes, and many more genes are differentially expressed in adipose tissue after TZD treatment than are affected in other tissues such as muscle and liver (Way et al., 2001; Rasouli et al., 2012). Proteins known to be upregulated in adipocytes by TZD treatment include glucose and fatty acid transporters (GLUT4, FATP1, CD36), fatty acid binding and sequestration proteins (aP2/FABP4, perilipin), glycerol channels (aquaporin 7), metabolic enzymes (LPL, PEPCK) and many others (Lefterova et al., 2010; Tontonoz & Spiegelman 2008; Henke, 2004; Nielsen et al., 2008). Also, selective PPARγ activation in mature adipocytes (by use of a gain-of-function fusion protein under the control of the aP2 promoter) improves insulin sensitivity in mice to a degree similar to that seen with TZD
treatment (Sugii et al., 2009). Several hypotheses have been advanced to explain how activation of PPARγ in adipose tissue could affect whole-body insulin sensitivity. Proposed mechanisms include 1) “fatty acid steal,” through which increased adipocyte differentiation and function redirects lipids from other tissues into adipose tissue for storage (Yki-Järvinen, 2004; Rasouli et al., 2005), and 2) altered secretion of adipose-derived cytokines and peptide hormones (Yki-Järvinen, 2004; Ferré, 2004).

“Fatty acid steal” and alleviation of lipotoxicity have been suggested to explain the effects of TZDs on both insulin sensitivity and β-cell function (Yki-Järvinen, 2004; Gastaldelli et al., 2007; Rasouli et al., 2007). After TZD treatment, declines in intracellular fat content correlate with increased insulin sensitivity in muscle and liver (Bajaj et al., 2010; Ravikumar et al., 2008), and declines in plasma free fatty acids correlate with increased insulin secretory response during oral glucose tolerance testing (Gastaldelli et al., 2007). However, the β-cell functional effects of the TZDs may also be mediated through direct PPARγ activation in the pancreas (Leahy, 2009). The pro-differentiation transcription factor PDX-1, which increases at a pivotal time during β-cell compensation in partially pancreatectomized rats (Moibi et al., 2007), is regulated by TZDs through a PPRE in its promoter (Gupta et al., 2008).

With regard to the second hypothesis (adipose tissue cross-talk through secretory products), TZDs increase plasma concentrations of adiponectin, an adipose-derived cytokine, in humans and rodents (Maeda et al., 2001). This takes place through changes in both adiponectin expression (Maeda et al., 2001; Iwaki et al., 2003) and secretion (Bodles et al., 2006). In humans, the increase in adiponectin concentration occurs early during the course of treatment (Hammarstedt et al., 2005). It has been documented in lean individuals as well as in obese, insulin-resistant, and diabetic subjects (Maeda et al., 2001; Yu et al., 2002), and its significance
in terms of insulin sensitization is not completely clear. However, adiponectin is known to improve hepatic insulin sensitivity in multiple species (Turer & Scherer, 2012), and in diabetic humans treated with pioglitazone, plasma adiponectin concentrations increase in proportion to decreasing liver fat and hepatic insulin resistance (Bajaj et al., 2004). In addition to its insulin-sensitizing effects, adiponectin has anti-apoptotic effects on β-cells *in vitro*, which appear to be mediated by reduction of ceramide concentrations (Turer & Scherer, 2012).

PPARγ-independent mechanisms of action for TZDs have also been proposed. For example, several members of this class bind to proteins found in the mitochondrial membrane (Colca et al., 2003; Bolten et al., 2007; Chen et al., 2012). In a recent investigation, a novel compound that bound mitochondria with similar affinity, but was a very weak PPARγ agonist, was shown to be as effective as other TZDs at lowering blood glucose in ob/ob mice (Chen et al., 2012). Also, in cell culture or isolated tissues, some TZDs induce Akt phosphorylation or activation of AMP-dependent kinase (a fuel-sensing regulatory enzyme) more rapidly than would be expected for a transcriptional effect (LeBrasseur et al., 2006; Bolten et al., 2007). Debate persists regarding the relative importance of PPARγ agonism and other mechanisms in the pharmacology of the TZDs. However, their agonism of the receptor is likely to account for at least some of their clinical properties.

**Adverse effects**

Dose-dependent adverse effects of the TZDs in both humans and animals include weight gain, mild, reversible anemia, and fluid retention (US FDA, 1999, Part 5; Aleo et al., 2003; Berria et al., 2007; Barnett, 2009.) Some of these effects are likely to be PPARγ-mediated; for example, fluid retention is thought to involve activation of PPARγ in the renal collecting duct,
with consequent changes in epithelial transport proteins (Pavlov et al., 2010). For others, the involvement of PPARγ has not been clarified. Hematocrit reduction (approximately 3%) occurs in the absence of a significant change in total body water in humans, and a mild bone marrow suppressive effect of the TZDs has been suggested (Berria et al., 2007). Weight gain typically amounts to 3-4 kg in obese humans over several months (Barnett, 2009). It occurs primarily as an increase in subcutaneous fat (Berria et al., 2007); although this has been ascribed to the adipogenic activities of PPARγ, it can be prevented with a portion-controlled diet (Martin et al., 2010).

TZD-induced fluid retention in humans may be manifest as peripheral or macular edema, or as an increased risk of congestive heart failure (Barnett, 2009). The latter was added as a boxed warning to the label for both pioglitazone and rosiglitazone during the postmarketing period (Starner et al., 2008). Other adverse effects recognized after marketing included distal limb fractures in women (both rosiglitazone and pioglitazone) (Loke et al., 2009), and a possible increase in the long-term risk of bladder cancer (pioglitazone) (Lewis et al., 2011). Bladder cancer was identified only in male rats during preclinical investigation of pioglitazone (US FDA, 1999, Part 6); as well, for other adverse effects, species susceptibility was variable (US FDA, 1999, Part 6).

In 2011, an increased incidence of cardiovascular adverse events was documented in rosiglitazone-treated patients, leading to the removal of rosiglitazone from non-US markets and its placement on a restricted prescription list in the US (Blind et al., 2010; US FDA, 2011). Thus, at the time of writing, pioglitazone is the only TZD available for general prescription.
Summary and implications

The potential benefits of TZDs in feline metabolic disorders formed the foundation for the work described in the remainder of this dissertation. First, in light of the pathophysiological similarities between human type 2 and feline DM, TZDs may be useful for treatment of DM in cats. Possible advantages of TZDs over current treatment options might include 1) dual effects on insulin sensitivity and β-cell function, 2) oral administration, 3) lag time to dissipation of effects, and 4) less risk of hypoglycemia. In the studies described here, pioglitazone was selected for investigation because of commercial availability, and obese cats were used as a model for drug effects due to their naturally occurring insulin resistance. As dose-concentration relationships for pioglitazone vary among species (US FDA, 1999, Part 6), a pharmacokinetic study was first conducted in order to select an appropriate dosage.

Second, the ability of TZDs to mobilize ectopic lipid may be of benefit in feline hepatic lipidosis. In humans, ¹H magnetic resonance spectroscopy (¹H MRS) is commonly used to measure hepatic triglyceride in clinical research (Schwenzer et al., 2009). ¹H MRS has been used in cats to measure triglyceride in muscle (Wilkins et al., 2004); in the work described in Chapter 5 of this dissertation, it was applied to measurement of feline liver fat. Historically, evaluation of the impact of drugs, diet composition, or other variables on liver lipid in cats has depended on histological or chemical examination of sequential liver biopsies (Biourge et al., 1994; Blanchard et al., 2002; Ibrahim et al., 2003). However, histological examination is not ideal for precise quantification, and biopsy is an invasive procedure that carries the risk of hemorrhage or iatrogenic damage to perihepatic structures (Armstrong & Blanchard, 2009). The main advantages of 1H MRS over previous approaches are its noninvasive nature and ability to accurately quantify tissue fat content (Schwenzer et al., 2009).
2.2 Figures and Legends

2.3 References


Chapter III

Pharmacokinetics of Pioglitazone in Lean and Obese Cats\textsuperscript{1}

3.1 Abstract

Pioglitazone is a thiazolidinedione insulin sensitizer that has shown efficacy in type 2 diabetes and non-alcoholic fatty liver disease in humans. It may be useful for treatment of similar conditions in cats. The purpose of this study was to investigate the pharmacokinetics of pioglitazone in lean and obese cats, in order to provide a foundation for assessment of its effects on insulin sensitivity and lipid metabolism. Pioglitazone was administered intravenously (median 0.2 mg/kg) or orally (3 mg/kg) to 6 healthy lean (3.96 $\pm$0.56 kg) and 6 obese (6.43 $\pm$0.48 kg) cats, in a two by two Latin Square design with a 4-week washout period. Blood samples were collected over 24 hours, and pioglitazone concentrations were measured via a validated HPLC assay. Pharmacokinetic parameters were determined using two-compartmental analysis for IV data and noncompartmental analysis for oral data. After oral administration, mean bioavailability was 55\%, \( t_{1/2} \) was 3.5 h, \( T_{\text{max}} \) was 3.6 h, \( C_{\text{max}} \) was 2131 ng/mL, and AUC\textsubscript{0-inf} was 15556 ng/mL*h. There were no statistically significant differences in pharmacokinetic parameters between lean and obese cats following either oral or intravenous administration. Systemic exposure to pioglitazone in cats after a 3 mg/kg oral dose approximates that observed in humans with therapeutic doses.

Key Words: Pioglitazone, diabetes, hepatic lipidosis, pharmacokinetics, thiazolidinediones

3.2 Introduction

The incidence of obesity in cats has risen dramatically in the last three decades. In both humans and cats, obesity leads to altered insulin secretion and sensitivity, and is a risk factor for type 2 diabetes mellitus (Strauss et al., 1974; Hoenig, 2002; Hoenig, 2006; Hoenig et al., 2007). Feline obesity also predisposes to dyslipidemia (Jordan et al., 2008), and to abnormal fat deposition in the liver (hepatic lipidosis) following a period of rapid weight loss. Hepatic lipidosis can occur in non-obese as well as obese cats, and its causes are not well understood (Brown et al., 2007); however, this condition has some pathophysiologic similarities to human non-alcoholic fatty liver disease (NAFLD), a chronic hepatopathy associated with obesity, insulin resistance, type 2 diabetes, and dyslipidemia (Pazak et al., 1998; Brown et al., 2007; Holt et al., 2006; Ahmed & Byrne, 2009).

Thiazolidinediones (TZDs) are a relatively new class of antidiabetic drugs approved for human use. They are labeled for adjunctive treatment of type 2 diabetes mellitus, and have recently shown therapeutic efficacy in patients with NAFLD (Ahmed & Byrne, 2009). TZDs are agonists of peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear transcription factor that is a key regulator of adipogenesis (Lehrke & Lazar, 2005). The effects of TZD administration are an increase in whole-body insulin sensitivity, and reduction of triglyceride content in non-adipose tissues such as liver and skeletal muscle (Lehrke & Lazar, 2005; Rasouli et al., 2005; Ravikumar et al., 2008; Bajaj et al., 2010).

Because the TZDs are effective for treatment of diabetes and NAFLD in people, they may be a promising therapeutic option for similar conditions in cats. In fact, treatment with darglitazone (an experimental TZD) for 6 weeks resulted in marked improvement in lipid and glucose metabolism in obese, insulin-resistant cats (Hoenig & Ferguson, 2000). The purpose of
this project was to describe the pharmacokinetics of the TZD pioglitazone in healthy lean and obese cats, in order to provide a foundation for the further study of this medication in cats with insulin resistance and hepatic lipid accumulation. A secondary purpose of the project was to investigate whether the pharmacokinetic parameters of pioglitazone are altered in obese cats, since potential clinical use of the drug would involve this subpopulation.

3.3 Materials and Methods

Animals

Six healthy lean (3.96 ±0.56 kg; median age 4 years, range 3-8 years) and six obese (6.43 ±0.48 kg; median age 5 years, range 4-8 years) neutered male Domestic Shorthair cats were used for this study. The cats were individually housed at the University of Illinois veterinary medical animal care facility. All procedures involving this study were approved by the university's Institutional Animal Care and Use Committee, and were conducted in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Food intake was adjusted to maintain a stable body weight.

Drug administration and sampling

Twenty-four to thirty-six hours before each study period, cats were sedated with tiletamine/zolazepam (Telazol™, Fort Dodge, IA, USA; 2-4 mg/kg intramuscularly based upon tiletamine) to facilitate placement of jugular and cephalic catheters. Throughout the study, catheter patency was maintained by flushing with sterile 0.38% sodium citrate.

In each study period, each cat was administered a single dose of pioglitazone either intravenously (1 mg/kg) or orally (3 mg/kg). After a washout period of at least 4 weeks, each cat
received the opposite formulation, in a two by two Latin Square design. Food was withheld for 12 hours before and 24 hours after drug administration.

For IV administration, pioglitazone HCl (Allichem LLC, Savage, MD, USA) was dissolved in medical grade dimethylsulfoxide (DMSO; 1 mg/100 µL) and diluted with fresh feline plasma and sterile 0.9% saline (10:45:45 DMSO:plasma:saline, v/v/v) to make a solution that contained 1 mg/mL pioglitazone base. Plasma had been collected the previous day from the same cat to which it was to be administered, and the plasma:saline mixture had been filtered through a 0.2 µM nylon syringe filter (Fisher Scientific, Pittsburgh, PA, USA) to remove particulates. The final pioglitazone solution was also filtered before administration to ensure sterility, and post-filtration samples were reserved for measurement of actual pioglitazone concentration to determine the IV dose administered to each cat. The solution was injected through a jugular or cephalic catheter over 5 minutes.

For oral administration, commercially available pioglitazone tablets (Actos™, Takeda Pharmaceuticals America, Inc., Deerfield, IL, USA) were crushed with a mortar and pestle and placed in gelatin capsules. Capsules were weighed before and after filling to determine the actual weight of ground tablet, and were administered with a small amount of canned cat food (Purina NF, Nestlé Purina, St. Louis, MO, USA). Cats were observed to be sure they had consumed the entire capsule.

Blood samples were obtained from the jugular catheter at 0, 5, 10, 15, 30 and 45 minutes, then at 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, and 24 hours after IV drug administration. After oral administration, samples were collected at 0, 15, 30 and 45 minutes, then at 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, and 24 hours. Samples were placed in EDTA microcentrifuge tubes and kept on ice until
centrifugation (10 minutes at 5000 x g), and the plasma was collected and stored at -20°C until assayed. Cats were monitored for clinical abnormalities throughout the sample collection period.

**Measurement of pioglitazone concentrations**

*a. Sample preparation*

Plasma pioglitazone concentrations were measured by high performance liquid chromatography (HPLC), using a modification of previously published methods for quantitation of TZDs in plasma (Zhong & Williams, 1996; Michels et al., 2000). All chemicals were HPLC grade. Methyl-4-hydroxybenzoate (Sigma Chemical Co., St. Louis, MO, USA) was used as an internal standard (IS). Standard solutions (1 mg/mL) were prepared by dissolving pioglitazone and IS separately in acetonitrile:water (50:50, v/v) and acetonitrile, respectively. The standard solutions were diluted with acetonitrile:water (50:50, v/v) to produce working standards, and the working standards of pioglitazone were added to blank feline plasma to prepare a calibration curve ranging from 2.5 ng-500 ng pioglitazone.

*b. Extraction method*

For extraction, IS (25 μL of 10 ng/μL), acetonitrile (100 μL), and ethyl acetate:hexane (90:10; 2 mL) were added to 200 or 250 μL of each standard or subject sample (250 μL for IV samples, and 200 μL for oral samples). After vortexing, the samples were centrifuged at 2,095 x g for 15 min. The organic layer was separated and evaporated to dryness under a stream of N₂ at 40°C, using silanized taper bottom tubes. The residue was resuspended in 100 μL of acetonitrile:water (50:50, v/v), vortexed, and transferred to a vial for HPLC analysis.
c. **Instrumentation**

Twenty-five µL of each sample or standard was injected onto an Agilent HPLC 1100 system with a Phenomenex Luna C18 (250 mm x 4.6 mm x 5 µM) column. Mobile phase consisted of 78% Solvent A (0.25% acetic acid in water) and 22% Solvent B (0.25% acetic acid in acetonitrile), changing to 60% Solvent A/40% Solvent B over 10 minutes. Flow rate was 1 mL/min., and the diode array detector was set for single wavelength acquisition at 269 nm with a 12 nm span. Under these conditions, pioglitazone and IS eluted at approximately 8 min and 11 min, respectively.

Pioglitazone concentration was determined from pioglitazone:IS peak area after linear regression analysis of the calibration standards using statistical software (Microsoft Excel 2008, Microsoft Corporation, Redmond, WA, USA). Each standard sample was run in duplicate.

d. **Method validation**

The quantitative HPLC method for pioglitazone was validated by examining the measurement of consistency of results (within-run and between-run), correlation (coefficient of determination of the standard curve), and extraction efficiency of the assay. The sample volume used for method validation was 200 µL. The within-run precision was calculated from similar responses of six repeats of three control samples (10, 100, 400 ng) in one run. The between-run precision was determined by comparing the calculated response (in ng/mL backfit of the standard curve) of the low (10 ng), middle (100 ng), and high (400 ng) control samples over three consecutive runs (total of six runs). The within-run and between-run accuracy was established by determining the ratio of calculated response to expected response for low (10 ng), middle (100 ng), and high (400 ng) control samples over six runs.
Standard curve correlation was measured by the mean coefficient of determination (r²) for six consecutive runs. The extraction efficiency was determined by comparing the response (in area) of low (10 ng), middle (100 ng), and high (400 ng) standards, and the internal standard (250 ng), spiked into blank plasma eluent before evaporation, to the response from equivalent extracted standards. The lower limit of detection (LOD) was calculated utilizing the analyte’s peak height compared to the baseline noise. By this method, the LOD was defined as the lowest concentration of analyte producing a peak greater than or equal to three times the baseline noise of the chromatogram. Additionally, the stability of pioglitazone (400 ng/200 µL, n=4 for each experiment) in acetonitrile:water (50:50, v/v) stored at room temperature, in sample matrix refrigerated (4°C) for 2 and 4 weeks, and in sample matrix frozen (−20°C) for 2 and 4 weeks was investigated. In this experiment, instrument responses from incubated samples were compared with those of freshly prepared samples using freshly prepared standard samples spiked with freshly prepared IS immediately before extraction.

Post-filtration IV samples of pioglitazone solution contained concentrations outside the range of the calibration curve. These samples were diluted 1:100 with blank feline plasma before extraction. Dilutional parallelism of the assay was confirmed by diluting duplicate samples of 1 mg/mL and 0.1 mg/mL pioglitazone 1:100 with blank feline plasma, and comparing assay results with expected concentrations. Mean ± SD accuracy was 96 ± 0.3% and 99 ± 8.1% for low and high concentrations, respectively, and coefficient of variation for both was <1%.

e. Pharmacokinetic and statistical analysis

Pharmacokinetic analysis was carried out using commercially available software (Phoenix WinNonlin 6.1, Pharsight Corporation, Cary, NC, USA). IV data were best fit by a two-compartmental model, and oral data were analyzed using a noncompartmental model.
Pharmacokinetic parameters calculated following IV administration included area under the plasma concentration-time curve from time 0 to infinity (AUC$_{0\text{-}\infty}$), elimination half-life ($t_{1/2\text{k}_{10}}$), distribution half-life ($t_{1/2\alpha}$), terminal elimination half-life ($t_{1/2\beta}$), y-intercept for the distribution phase (A), y-intercept for the elimination phase (B), maximum plasma concentration ($C_{\text{max}}$), systemic clearance ($Cl_s$), area under the moment curve from time 0 to infinity (AUMC$_{0\text{-}\infty}$), volume of distribution at steady state ($V_{ss}$), volume of distribution of the central compartment ($V_c$), and volume of distribution of the terminal elimination phase ($V_\beta$). Pharmacokinetic parameters calculated following oral administration included oral bioavailability ($F$), terminal elimination rate constant ($\lambda z$), terminal elimination half-life ($t_{1/2\lambda z}$), area under the plasma concentration-time curve from time 0 to infinity (AUC$_{0\text{-}\infty}$), time to maximum plasma concentration ($T_{\text{max}}$), maximum plasma concentration ($C_{\text{max}}$), oral clearance ($Cl/F$), and mean residence time (MRT).

Pharmacokinetic parameters from lean and obese cats were compared using a statistical software package (GraphPad Prism Version 5.00 for Windows, Graph Pad Software, San Diego, CA, USA). A Kolgorov-Smirnov test was used to assess normality of the data, and an F-test was used to compare variances. Those parameters that were normally distributed in both lean and obese cats were compared using an unpaired Student's t test with Welch's correction for unequal variances, if necessary; those with nonnormal distribution in either lean or obese cats were compared using a Mann-Whitney U test. For all comparisons, hypothesis testing was two-tailed, and p-values less than 0.05 were considered significant.
3.4 Results

The HPLC diode array detection method reported here readily detects pioglitazone in plasma, with a LOD of approximately 2.5 ng/mL. Mean ± SD extraction efficiency was 93 ± 2.8%, 94 ± 6.0%, and 88 ± 5.0% at quantities of 10, 100, and 400 ng pioglitazone, respectively. Mean ± SD within-run accuracy was 99 ± 7.4%, 103 ± 7.4 %, and 104 ± 1.4 % for low, mid-range, and high concentrations, respectively. Corresponding coefficients of variation were 7.5, 7.2, and 1.3%. Mean ± SD between-run accuracy was 101 ± 5.4%, 104 ± 4.3%, and 101 ± 4.1% for low, mid-range, and high concentrations, respectively. Corresponding coefficients of variation were 5.4, 4.1, and 4.0%.

Results of HPLC analysis demonstrated 103 ± 1% (SD) recovery of pioglitazone stored in acetonitrile:water (50:50, v/v) at room temperature for 24 hr. The stability of pioglitazone in feline plasma samples stored refrigerated and frozen (-20 °C) for 2 weeks was 104 ± 2% (SD) and 104 ± 4% (SD), respectively. The stability of pioglitazone in feline plasma samples stored refrigerated and frozen (-20 °C) for 4 weeks was 108 ± 3.6% (SD) and 106 ± 6.9% (SD), respectively.

Plasma concentration-time curves after IV administration of pioglitazone to lean and obese cats are shown in Figure 3.1. Drug loss occurred during the final filtration step in the preparation of all IV pioglitazone solutions; as a result, the actual dosage of pioglitazone administered intravenously, as measured in post-filtration samples of prepared solution, ranged from 0.08-0.8 mg/kg, with a median of 0.17 mg/kg. For comparison of the shapes of plasma concentration-time curves among individual cats and between lean and obese cats, concentrations obtained after IV administration were normalized to a dosage of 1 mg/kg. However, all data analysis, including calculation of pharmacokinetic parameters, was performed
using actual (measured) pioglitazone concentrations. The latter procedures assume linearity of pioglitazone pharmacokinetics in cats; although this assumption has not been confirmed, linear pharmacokinetics have been demonstrated for pioglitazone in dogs at dosages of 0.1-3 mg/kg (Maeshiba, 1997), in rats at dosages of 0.5-30 mg/kg (Maeshiba, 1997), and in humans at doses of 7.5-45 mg (0.1-0.6 mg/kg for a 70 kg human, Eckland & Danhof, 2000). Body weights at the time of IV administration, actual dosages of pioglitazone administered intravenously, and pharmacokinetic parameters after IV administration are shown for lean and obese cats in Tables 3.1 and 3.2, respectively.

Plasma concentration-time curves after oral administration of pioglitazone to lean and obese cats are shown in Figure 3.2. Oral administration of 3 mg/kg pioglitazone resulted in rapid absorption in 11/12 cats, although the extent of absorption was variable. In one lean cat, pioglitazone was not detectable in plasma until 2 hours post-administration, after which concentrations increased to a peak at 12 h. Measurement of concentrations was repeated on a different aliquot of the samples for verification, with the same result. As this administration in this cat was considered an outlier, and as no data between 14 and 24 h were available for calculation of the terminal elimination half-life and AUC\textsubscript{0-inf}, oral pharmacokinetic parameters from this cat were not included in calculations of means or medians for statistical comparison. Body weights at the time of oral administration, actual dosages of pioglitazone administered orally, and pharmacokinetic parameters after oral administration are shown for lean and obese cats in Tables 3.3 and 3.4, respectively.

No significant difference was identified for any parameter between lean and obese cats. P-values for comparison of each parameter between lean and obese cats are shown for IV and
oral administration in Tables 3.5 and 3.6, respectively, as are mean ± standard deviation or median and range of pharmacokinetic parameters for all cats (lean and obese cats combined).

Dark red urine was noted in the litterbox of one obese cat after the cat received IV pioglitazone solution (4.6 mL solution; 0.18 mg/mL pioglitazone in DMSO). The cat’s mucous membranes were pink, and attitude and clinical status remained within normal limits throughout the sample collection period. No other abnormalities were noted in association with pioglitazone administration in any of the cats, or during subsequent daily observation of the pigmenturic cat.

3.5 Discussion

The pharmacokinetics of pioglitazone have been characterized in rats, dogs, monkeys, and humans (Krieter et al., 1994; Maeshiba et al., 1997; Eckland & Danhof, 2000, Budde et al., 2003; Kalliokoski et al., 2007) and have recently been described in horses (Wearn et al., 2010). Data regarding bioavailability, volume of distribution, and systemic clearance after IV administration are available from a single study in each species except the horse, in which only the oral form of the drug was given due to lack of a commercial IV formulation.

Mean bioavailability of pioglitazone in the cats of this study (approximately 55%) was lower than that reported for other species (85%, 94%, and 81%, and 83% in rats, dogs, monkeys, and humans, respectively (Maeshiba et al., 1997; Eckland & Danhof, 2000). Bioavailability appeared to vary substantially among cats; however, a 95% confidence interval for the bioavailability data (45-67%) is similar in width to that reported for humans (74-93%; Eckland & Danhof, 2000). Estimates of interindividual variation in the bioavailability of pioglitazone in other animals are not available, as reported bioavailability data for rats, dogs, and primates were
calculated from a mean dose and mean AUC$_{0\text{-}\text{inf}}$ of 3-5 animals of each species (Maeshiba et al., 1997).

Differences in bioavailability of the same drug among individuals or groups can be caused by properties of the drug formulation, or by differences in physiological factors such as gastrointestinal transit time, pH of the gastrointestinal contents, intestinal transport proteins, and first-pass metabolism. In the laboratory animals mentioned above, bioavailability data were generated using an oral solution of pioglitazone in citric acid. This may account for the difference in bioavailability between these species and the cats studied here. In humans, the marketed form of the drug is a rapidly disintegrating tablet, although bioequivalent encapsulated forms were used in some clinical studies (Xue et al., 2003). Because dissolution, rather than disintegration, is the limiting step for absorption of most drugs from solid dosage forms (Baggott, 2001), the bioavailability of pioglitazone in rapidly disintegrating tablets and in the gelatin capsules used in this study is expected to be similar. Thus, the lower mean bioavailability of the drug in cats compared to humans probably reflects the influence of various species-related physiologic factors. Also, extent of absorption in cats dosed with the commercial tablet form of the drug is likely to approximate values reported in this study.

Absorption of orally administered pioglitazone was rapid in most cats, with a mean $T_{\text{max}}$ (3.6 h) similar to those of rats (4.0 ± 0.0 h), monkeys (4.3 ± 3.2 h), and humans (mean 1.5 or 2 h; range 0.5-3 h) (Maeshiba et al., 1997; Eckland & Danhof, 2000). The high $T_{\text{max}}$ (12 h post-administration) noted in a single lean cat was suspected to be due to retention of the capsule in the esophagus (despite administration with a small amount of canned food) or delayed gastric emptying, rather than an intrinsic difference in intestinal absorption. In this cat, the shape of the
plasma concentration-time curve was not consistent with slow absorption over the entire dosing period, and $C_{\text{max}}$ was comparable to that of the other lean cats.

Other than the small amount of canned food given to facilitate dosing, animals in this study were fasted prior to drug administration and during the sample collection period. In humans, administering pioglitazone in the fed state delayed absorption slightly (mean $T_{\text{max}}$ of 3-4 h rather than 2 h), but did not affect the extent of absorption (Eckland & Danhof, 2000).

The volume of distribution at steady state ($V_{\text{ss}}$) of pioglitazone after IV administration in cats (median 0.36 L/kg, range 0.27-0.63) is similar to the mean $V_{\text{ss}}$ in humans (0.25 ± 0.03 L/kg (EMEA 2004), rats, dogs, and monkeys (0.22 ± 0.005 L/kg, 0.47 ± 0.05 L/kg, and 0.31 ± 0.07 L/kg, respectively (Maeshiba 1997). In humans and rodents, pioglitazone is >97% bound to albumin, which limits its distribution outside the plasma compartment, despite its high lipophilicity (Eckland & Danhof, 2000). Although the extent of plasma protein binding of pioglitazone in cats has not been studied, its median volume of distribution suggests that it also remains primarily in the plasma compartment in cats, and may also be highly protein-bound in this species.

Systemic clearance of pioglitazone in cats (mean 114 ± 37 mL/kg/h) is increased relative to systemic clearance in humans and rats (both approximately 60 mL/kg/h (Maeshiba et al., 1997; Eckland & Danhof, 2000), and decreased relative to systemic clearance in dogs (329 ± 43 mL/kg/h (Maeshiba et al., 1997). In humans, pioglitazone is metabolized primarily by hepatic CYP2C8 and CYP3A4, with only a small amount of parent drug excreted in the urine (EMEA, 2004). At least three hydroxylated or oxidized metabolites (M-III, M-IV, and M-V) have been identified in human serum; of these, two (M-III and M-IV) possess pharmacological activity (Zhong & Williams, 1996; Eckland & Danhof, 2000). Hepatic metabolism of pioglitazone is also
the predominant mechanism of clearance in rodents, dogs, and monkeys (Maeshiba et al., 1997). However, major metabolites, their rates of formation, and their routes of excretion differ among species (Maeshiba et al., 1997; Shen et al., 2003).

In a study of the pharmacokinetics of troglitazone, a compound structurally similar to pioglitazone, in cats (Michels et al., 2000), no parent drug was detected in the urine of dosed cats over a 72-h period. Based on these data and on the data from other species, it is probable that pioglitazone is metabolized by the liver in cats. The current study did not provide any direct information about pathways of pioglitazone metabolism in the cat; no obvious metabolites were identified in HPLC analysis of samples from dosed animals. This may have been a result of the fact that the HPLC methodology used was optimized for detection of parent drug, as was the assay in the above-referenced study of troglitazone (which also did not detect metabolites). The reason for the increased clearance of pioglitazone in cats relative to humans, and decreased clearance in cats relative to dogs, is not clear at this time due to the paucity of information regarding pioglitazone metabolism in cats.

The mean oral elimination half-life (3.45 ± 0.93 h) for pioglitazone in the cats of this study is shorter than the mean 5.8 h (range: 3 to 9 h) reported for humans (Eckland & Danhof, 2000), and than the mean 9.94 ± 4.57 h reported for horses (Wearn et al., 2010). As a consequence, steady-state concentrations of pioglitazone in cats are likely to be achieved after a single administration. In humans, the pharmacologically active metabolites of pioglitazone have significantly longer half-lives than the parent drug (26-28 h for M-III and M-IV (Eckland & Danhof, 2000)). The same is true in rats, in which pioglitazone has a half-life of 2.6 h (Maeshiba et al., 1997), but produces a therapeutic effect when dosed every 24 hours (Ikeda et al., 1990; Sugiyama et al., 1990). As discussed above, the metabolites of pioglitazone in cats and their
half-lives have not been studied, and the effects of pioglitazone, like those of the glucocorticoids, are mediated through changes in gene expression. For these reasons, it is difficult to predict the impact of a shorter half-life for pioglitazone on the duration of its biological activity in cats.

The mean AUC$_{0\text{-}\text{inf}}$ (15556 ± 5556 ng/mL*h) achieved by administration of 3 mg/kg oral pioglitazone in the cats of this study is within the range of exposures associated with therapeutic efficacy in humans, and the mean $C_{\text{max}}$ (2131 ± 607 ng/mL) is slightly higher. In humans, the therapeutic dose range is 15-45 mg; mean maximum plasma concentrations of pioglitazone in humans receiving doses of 15 and 45 mg have been reported to be 617±129 and 1552 ± 708 ng/mL, respectively (Kalliokoski et al., 2007; Eckland & Danhof, 2000). Areas under the plasma concentration-time curve for these doses were 6244 ± 1909 and 14071 ± 5727 ng/mL*h, respectively. The exposure-response relationship for pioglitazone in rats is similar to the relationship in humans (Maeshiba et al., 1997; Ikeda et al., 1990). Assuming that this is also true for cats, data from this study indicate that 1-3 mg/kg would be an appropriate oral dosage for efficacy assessment of pioglitazone in cats.

The safety of pioglitazone in cats after multiple dosing has not yet been evaluated, although no adverse effects were seen here after oral administration of a single 3 mg/kg dose of the drug. Dark red urine was noted in one cat after IV administration of pioglitazone solution. As noted previously, pioglitazone is lipophilic and practically insoluble in water, and an IV formulation is not commercially available. In pharmacokinetic studies using rodents, dogs, and monkeys, pioglitazone was dissolved in DMSO for IV administration (Maeshiba et al., 1997); in humans, it was dissolved in 30% propylene glycol and a maximum dose of 5 mg (0.07 mg/kg for a 70 kg human) was given as an infusion over 2 hours (Eckland & Danhof, 2000). In the study reported here, pioglitazone was dissolved in a plasma:saline:DMSO solution for IV
administration. This strategy had been previously employed in the study by Michels et al. involving the lipid-soluble drug troglitazone (Michels et al., 2000). In that investigation, DMSO comprised 25% of the final solution by volume, and transient hemolysis was noted in the three cats to which the drug was given intravenously. DMSO has been documented to cause hemolysis, hematuria, and methemoglobinemia in cats; severity increases with increased concentration of DMSO (DiStefano & Klahn, 1965). The percentage of DMSO in the pioglitazone solution administered here was decreased to 10% in an attempt to avoid hemolysis.

No statistically significant differences were identified in the pharmacokinetic parameters for IV or oral pioglitazone between lean and obese cats. Elimination half-life after oral administration tended to be shorter in lean cats, and this difference approached statistical significance. As the sample size in this study was initially designed to detect differences in AUC$_{0\text{-}\text{inf}}$, differences in half-life or other parameters may not have been apparent due to insufficient statistical power. In humans, no direct comparisons of pharmacokinetic parameters for pioglitazone between lean and obese individuals have been published. However, no obvious differences from healthy volunteers have been seen in the pharmacokinetics of pioglitazone in patients with type 2 diabetes, many of whom are obese, and no dosage adjustment is recommended for the drug in obesity (EMEA, 2004).

All the cats in this study were male. Differences in the pharmacokinetics of pioglitazone between male and female rats have been reported; in a study by Fujita et al. (2003), mean C$_{\text{max}}$, AUC$_{0\text{-}\text{inf}}$, and $t_{1/2}$ were increased by 20-50% in intact female rats. A slightly increased C$_{\text{max}}$ and AUC$_{0\text{-}\text{inf}}$ for pioglitazone have also been seen in human females, but dose in these studies was not normalized for the lower body weight of the female subjects, and the differences were not considered to be clinically relevant (EMEA, 2004).
In conclusion, pioglitazone is rapidly absorbed in cats after oral administration, with a mean bioavailability of 55%. Volume of distribution of the drug after IV administration is similar to that seen in other species, while clearance is increased and half-life is decreased relative to humans. Maximum plasma concentrations and total drug exposure, as measured by area under the plasma concentration-time curve, are variable after a 3 mg/kg oral administration, but approximate those associated with therapeutic efficacy in humans. Further study will be necessary in order to establish an exposure-response relationship for pioglitazone in cats with insulin resistance and hepatic lipid accumulation, and to evaluate its efficacy in feline patients with type 2 diabetes mellitus or hepatic lipidosis.
3.6 Figures and Legends

Figure 3.1 Mean plasma concentration (±SD) vs. time for lean (n=6) and obese (n=6) cats after IV administration of pioglitazone. Concentrations were normalized to a dosage of 1 mg/kg.
**Figure 3.2** Mean plasma concentration (±SD) vs. time for lean (n=5) and obese (n=6) cats after oral administration of 3 mg/kg pioglitazone.
Table 3.1 Pharmacokinetic parameters for pioglitazone following IV administration in lean cats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cat 1</th>
<th>Cat 2</th>
<th>Cat 3</th>
<th>Cat 4</th>
<th>Cat 5</th>
<th>Cat 6</th>
<th>Mean ± SD, or median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>4.48</td>
<td>4.3</td>
<td>3.6</td>
<td>3.43</td>
<td>3.36</td>
<td>4.6</td>
<td>4.0 (3.4-4.6)</td>
</tr>
<tr>
<td>Dosage (mg/kg)</td>
<td>0.84</td>
<td>0.16</td>
<td>0.09</td>
<td>0.09</td>
<td>0.14</td>
<td>0.18</td>
<td>0.15 (0.09-0.84)</td>
</tr>
<tr>
<td>AUC₀⁻infinity (h*ng/ml)</td>
<td>4627</td>
<td>1319</td>
<td>841</td>
<td>1231</td>
<td>1211</td>
<td>2027</td>
<td>1876 ± 1402</td>
</tr>
<tr>
<td>t₁/₂ k₁₀ (h)</td>
<td>1.73</td>
<td>1.40</td>
<td>0.46</td>
<td>1.05</td>
<td>0.41</td>
<td>0.90</td>
<td>0.99 ± 0.52</td>
</tr>
<tr>
<td>t₁/₂ α (h)</td>
<td>0.44</td>
<td>0.12</td>
<td>0.06</td>
<td>0.24</td>
<td>0.08</td>
<td>0.09</td>
<td>0.17 ± 0.15</td>
</tr>
<tr>
<td>t₁/₂ β (h)</td>
<td>2.64</td>
<td>2.18</td>
<td>2.32</td>
<td>2.97</td>
<td>2.56</td>
<td>3.07</td>
<td>2.62 ± 0.35</td>
</tr>
<tr>
<td>A (ng/ml)</td>
<td>771</td>
<td>245</td>
<td>1049</td>
<td>570</td>
<td>1751</td>
<td>1145</td>
<td>910 (245-1751)</td>
</tr>
<tr>
<td>B (ng/ml)</td>
<td>1087</td>
<td>406</td>
<td>224</td>
<td>242</td>
<td>276</td>
<td>425</td>
<td>341 (224-1087)</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>1857</td>
<td>652</td>
<td>1273</td>
<td>812</td>
<td>2027</td>
<td>1570</td>
<td>1365 ± 556</td>
</tr>
<tr>
<td>Cl (mL/kg/h)</td>
<td>182</td>
<td>123</td>
<td>102</td>
<td>73</td>
<td>113</td>
<td>90</td>
<td>113 ± 38</td>
</tr>
<tr>
<td>AUMC h<em>h</em>ng/ml</td>
<td>16064</td>
<td>4009</td>
<td>2511</td>
<td>4512</td>
<td>3796</td>
<td>8353</td>
<td>6541 ± 5065</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>3.47</td>
<td>3.04</td>
<td>2.99</td>
<td>3.67</td>
<td>3.13</td>
<td>4.12</td>
<td>3.40 ± 0.44</td>
</tr>
<tr>
<td>Vss (L/kg)</td>
<td>0.63</td>
<td>0.38</td>
<td>0.31</td>
<td>0.27</td>
<td>0.35</td>
<td>0.37</td>
<td>0.36 (0.27-0.63)</td>
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<tr>
<td>V₁ (L/kg)</td>
<td>0.45</td>
<td>0.25</td>
<td>0.07</td>
<td>0.11</td>
<td>0.07</td>
<td>0.12</td>
<td>0.18 ± 0.15</td>
</tr>
<tr>
<td>V₂ (L/kg)</td>
<td>0.18</td>
<td>0.13</td>
<td>0.24</td>
<td>0.16</td>
<td>0.29</td>
<td>0.25</td>
<td>0.21 ± 0.06</td>
</tr>
</tbody>
</table>
Table 3.2 Pharmacokinetic parameters for pioglitazone following IV administration in obese cats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cat 1</th>
<th>Cat 2</th>
<th>Cat 3</th>
<th>Cat 4</th>
<th>Cat 5</th>
<th>Cat 6</th>
<th>Mean ± SD, or median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>6.6</td>
<td>5.53</td>
<td>6.25</td>
<td>6.7</td>
<td>6.85</td>
<td>6.64</td>
<td>6.4 (5.5-6.9)</td>
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<tr>
<td>Dosage (mg/kg)</td>
<td>0.23</td>
<td>0.08</td>
<td>0.12</td>
<td>0.29</td>
<td>0.17</td>
<td>0.25</td>
<td>0.2 (0.08-0.29)</td>
</tr>
<tr>
<td>AUC(_{0-\text{inf}}) (h*ng/ml)</td>
<td>1703</td>
<td>954</td>
<td>883</td>
<td>2920</td>
<td>2854</td>
<td>1484</td>
<td>1800 ± 898</td>
</tr>
<tr>
<td>t(<em>{1/2}) k(</em>{10}) (h)</td>
<td>1.13</td>
<td>1.30</td>
<td>0.96</td>
<td>0.84</td>
<td>1.68</td>
<td>0.82</td>
<td>1.12 ± 0.33</td>
</tr>
<tr>
<td>t(_{1/2}) α (h)</td>
<td>0.16</td>
<td>0.12</td>
<td>0.12</td>
<td>0.13</td>
<td>0.25</td>
<td>0.09</td>
<td>0.14 ± 0.16</td>
</tr>
<tr>
<td>t(_{1/2}) β (h)</td>
<td>3.23</td>
<td>2.32</td>
<td>1.88</td>
<td>2.84</td>
<td>4.14</td>
<td>1.87</td>
<td>2.71 ± 0.88</td>
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<tr>
<td>A (ng/ml)</td>
<td>720</td>
<td>235</td>
<td>336</td>
<td>1782</td>
<td>741</td>
<td>734</td>
<td>727 (235-1782)</td>
</tr>
<tr>
<td>B (ng/ml)</td>
<td>329</td>
<td>273</td>
<td>303</td>
<td>628</td>
<td>434</td>
<td>515</td>
<td>382 (273-628)</td>
</tr>
<tr>
<td>C(_{\text{max}}) (ng/ml)</td>
<td>1048</td>
<td>508</td>
<td>639</td>
<td>2409</td>
<td>1175</td>
<td>1248</td>
<td>1171 ± 675</td>
</tr>
<tr>
<td>Cl (mL/kg/h)</td>
<td>137</td>
<td>85</td>
<td>131</td>
<td>99</td>
<td>59</td>
<td>169</td>
<td>113 ± 40</td>
</tr>
<tr>
<td>AUMC h<em>h</em>ng/ml</td>
<td>7190</td>
<td>3063</td>
<td>2250</td>
<td>10623</td>
<td>15548</td>
<td>3751</td>
<td>7071 ± 5193</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>4.22</td>
<td>3.21</td>
<td>2.55</td>
<td>3.64</td>
<td>5.45</td>
<td>2.53</td>
<td>3.60 ± 1.11</td>
</tr>
<tr>
<td>V(_{ss}) (L/kg)</td>
<td>0.58</td>
<td>0.27</td>
<td>0.33</td>
<td>0.36</td>
<td>0.32</td>
<td>0.43</td>
<td>0.35 (0.27-0.58)</td>
</tr>
<tr>
<td>V1 (L/kg)</td>
<td>0.22</td>
<td>0.16</td>
<td>0.18</td>
<td>0.12</td>
<td>0.14</td>
<td>0.20</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>V2 (L/kg)</td>
<td>0.36</td>
<td>0.11</td>
<td>0.15</td>
<td>0.24</td>
<td>0.18</td>
<td>0.23</td>
<td>0.21 ± 0.09</td>
</tr>
<tr>
<td>Parameter</td>
<td>Cat 1</td>
<td>Cat 2</td>
<td>Cat 3</td>
<td>Cat 4</td>
<td>Cat 5</td>
<td>Cat 6*</td>
<td>Mean ±SD, or median (range)</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>4.56</td>
<td>4.25</td>
<td>3.6</td>
<td>3.47</td>
<td>3.39</td>
<td>4.58</td>
<td>3.98 ± 0.55</td>
</tr>
<tr>
<td>Total dose (mg)</td>
<td>15</td>
<td>12.9</td>
<td>10.8</td>
<td>10.4</td>
<td>10.6</td>
<td>13.7</td>
<td>12.2 ± 1.9</td>
</tr>
<tr>
<td>Dosage (mg/kg)</td>
<td>3.29</td>
<td>3.04</td>
<td>3.00</td>
<td>3.00</td>
<td>3.13</td>
<td>2.99</td>
<td>3.08 ± 0.05</td>
</tr>
<tr>
<td>F (%)</td>
<td>86</td>
<td>63</td>
<td>49</td>
<td>52</td>
<td>46</td>
<td>NC</td>
<td>59 ± 16</td>
</tr>
<tr>
<td>$\lambda_z$ (1/h)</td>
<td>0.27</td>
<td>0.27</td>
<td>0.25</td>
<td>0.18</td>
<td>0.26</td>
<td>NC</td>
<td>0.26 (0.18-0.27)</td>
</tr>
<tr>
<td>$t_{1/2}$ $\lambda_z$ (h)</td>
<td>2.54</td>
<td>2.60</td>
<td>2.76</td>
<td>3.89</td>
<td>2.64</td>
<td>NC</td>
<td>2.64 (2.54-3.89)</td>
</tr>
<tr>
<td>$\text{AUC}_{0-\text{inf}}$ (h*ng/ml)</td>
<td>15906</td>
<td>15298</td>
<td>14500</td>
<td>21774</td>
<td>12821</td>
<td>NC</td>
<td>16060 ± 3398</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>5</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>12</td>
<td>5 (2-5)</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>2423</td>
<td>3012</td>
<td>2125</td>
<td>2625</td>
<td>2025</td>
<td>2301</td>
<td>2442 ± 398</td>
</tr>
<tr>
<td>$\text{CL}_F$ (ml/kg/h)</td>
<td>207</td>
<td>198</td>
<td>207</td>
<td>138</td>
<td>244</td>
<td>NC</td>
<td>199 ± 38</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>7.41</td>
<td>4.71</td>
<td>6.42</td>
<td>8.70</td>
<td>5.92</td>
<td>NC</td>
<td>6.6 ±1.5</td>
</tr>
</tbody>
</table>
**Table 3.4** Pharmacokinetic parameters for pioglitazone following oral administration in obese cats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cat 1</th>
<th>Cat 2</th>
<th>Cat 3</th>
<th>Cat 4</th>
<th>Cat 5</th>
<th>Cat 6</th>
<th>Mean±SD, or median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>6.6</td>
<td>5.5</td>
<td>6.35</td>
<td>6.6</td>
<td>6.67</td>
<td>6.72</td>
<td>6.6 (5.5-6.72)</td>
</tr>
<tr>
<td>Total dose (mg)</td>
<td>19.7</td>
<td>16.7</td>
<td>19.1</td>
<td>19.9</td>
<td>19.9</td>
<td>20</td>
<td>19.2 ± 1.3</td>
</tr>
<tr>
<td>Dosage (mg/kg)</td>
<td>2.98</td>
<td>3.04</td>
<td>3.01</td>
<td>3.02</td>
<td>2.98</td>
<td>2.98</td>
<td>3.00 ± 0.01</td>
</tr>
<tr>
<td>F (%)</td>
<td>83</td>
<td>50</td>
<td>28</td>
<td>36</td>
<td>55</td>
<td>60</td>
<td>52 ± 19</td>
</tr>
<tr>
<td>λz (1/h)</td>
<td>0.21</td>
<td>0.13</td>
<td>0.22</td>
<td>0.20</td>
<td>0.14</td>
<td>0.20</td>
<td>0.20 (0.13-0.22)</td>
</tr>
<tr>
<td>t1/2 λz (h)</td>
<td>3.24</td>
<td>5.38</td>
<td>3.21</td>
<td>3.38</td>
<td>4.84</td>
<td>3.48</td>
<td>3.43 (3.21-5.38)</td>
</tr>
<tr>
<td>AUC₀-inf (h*ng/ml)</td>
<td>18037</td>
<td>17724</td>
<td>6404</td>
<td>10837</td>
<td>27102</td>
<td>10716</td>
<td>15137 ± 7383</td>
</tr>
<tr>
<td>T_max (h)</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>3.5 (2-4)</td>
</tr>
<tr>
<td>C_max (ng/ml)</td>
<td>2223</td>
<td>1935</td>
<td>1112</td>
<td>1275</td>
<td>2918</td>
<td>1764</td>
<td>1871 ± 658</td>
</tr>
<tr>
<td>Cl_F (ml/kg/h)</td>
<td>165</td>
<td>171</td>
<td>470</td>
<td>278</td>
<td>110</td>
<td>278</td>
<td>245 ± 129</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>6.57</td>
<td>9.56</td>
<td>6.01</td>
<td>7.18</td>
<td>9.40</td>
<td>5.73</td>
<td>7.4 ± 1.7</td>
</tr>
</tbody>
</table>
**Table 3.5** P-values for comparison of pharmacokinetic parameters between lean and obese cats after single intravenous administration of pioglitazone, and mean ± SD or median (range) of each value for all cats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>p-value (lean vs. obese)</th>
<th>Mean ± SD, or median (range) for all cats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td><strong>0.002</strong></td>
<td>--</td>
</tr>
<tr>
<td>Dosage (mg/kg)</td>
<td>0.688</td>
<td>0.17 (0.08-0.84)</td>
</tr>
<tr>
<td>AUC (h*ng/ml)</td>
<td>0.913</td>
<td>1838 ± 1123</td>
</tr>
<tr>
<td>t1/2 K10 (h)</td>
<td>0.615</td>
<td>1.06 ± 0.42</td>
</tr>
<tr>
<td>t1/2 α (h)</td>
<td>0.685</td>
<td>0.12 (0.06-0.44)</td>
</tr>
<tr>
<td>t1/2 β (h)</td>
<td>0.821</td>
<td>2.67 ± 0.64</td>
</tr>
<tr>
<td>A (ng/ml)</td>
<td>0.485</td>
<td>840 ± 517</td>
</tr>
<tr>
<td>B (ng/ml)</td>
<td>0.485</td>
<td>429 ± 240</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>0.599</td>
<td>1268 ± 598</td>
</tr>
<tr>
<td>Cl (mL/kg/h)</td>
<td>0.983</td>
<td>114 ± 37</td>
</tr>
<tr>
<td>AUMC h*ng/ml</td>
<td>0.862</td>
<td>4261 (2250-16064)</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>0.696</td>
<td>3.5 ± 0.81</td>
</tr>
<tr>
<td>Vss (L/kg)</td>
<td>1.000 (Gaussian approximation)</td>
<td>0.36 (0.27-0.63)</td>
</tr>
<tr>
<td>V1 (L/kg)</td>
<td>0.514</td>
<td>0.17 ± 0.10</td>
</tr>
<tr>
<td>V2 (L/kg)</td>
<td>0.501</td>
<td>0.21 ± 0.07</td>
</tr>
</tbody>
</table>
Table 3.6  P-values for comparison of pharmacokinetic parameters between lean and obese cats after single oral administration of pioglitazone, and mean ± SD or median (range) of each value for all cats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>p-value (lean vs. obese)</th>
<th>Mean±SD, or median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>0.002</td>
<td>--</td>
</tr>
<tr>
<td>Dosage (mg/kg)</td>
<td>0.195</td>
<td>3.00 (2.98-3.29)</td>
</tr>
<tr>
<td>F (%)</td>
<td>0.527</td>
<td>55 ± 18</td>
</tr>
<tr>
<td>λ z (1/h)</td>
<td>0.055</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td>t1/2 λ z (h)</td>
<td>0.052</td>
<td>3.45 ± 0.93</td>
</tr>
<tr>
<td>AUC (h*ng/ml)</td>
<td>0.792</td>
<td>15556 ± 5666</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>0.351</td>
<td>3.64 ± 1.12</td>
</tr>
<tr>
<td>C_{max} (ng/ml)</td>
<td>0.125</td>
<td>2131 ± 607</td>
</tr>
<tr>
<td>Cl_F (ml/kg/h)</td>
<td>0.432</td>
<td>224 ± 97</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>0.446</td>
<td>7.06 ± 1.6</td>
</tr>
</tbody>
</table>
3.7 References


4.1 Abstract

Pioglitazone is a thiazolidinedione (TZD) insulin sensitizer approved for use in human type 2 diabetes mellitus. In diabetic humans, this drug improves both glycemic control and lipid metabolism, and also has beneficial effects on pancreatic β-cell function. The most common form of diabetes in cats, like type 2 diabetes in humans, is characterized by peripheral insulin resistance and insulin secretory defects; therapeutic options for this disease in cats are limited, and additional treatment choices would be beneficial. The purpose of this project was to evaluate the metabolic effects of pioglitazone in obese, insulin-resistant cats, to assess its potential for future use in feline diabetes mellitus. Oral pioglitazone (Actos™) was administered at 1 and 3 mg/kg to 12 obese cats, in a placebo-controlled 3-way crossover design. Effects on insulin sensitivity, glucose clearance, and non-esterified fatty acid clearance were determined using intravenous glucose tolerance testing (IVGTT); additionally, effects on energy expenditure were measured via indirect calorimetry. After 6 weeks of daily dosing, 3 mg/kg pioglitazone significantly improved insulin sensitivity, reduced insulin area under the curve during IVGTT, and lowered serum triglyceride and cholesterol concentrations in the obese cats. No changes in energy expenditure were noted, and no adverse effects attributable to pioglitazone were evident in the otherwise healthy obese cats at this dosage and duration. Results of this study support a positive effect of pioglitazone on insulin sensitivity and lipid metabolism in obese cats, and
suggest that further evaluation of the drug in cats with diabetes or other metabolic disorders may be warranted.

4.2 Introduction

Diabetes mellitus is a disorder of carbohydrate, lipid, and protein metabolism arising from absolute or relative insulin deficiency. It occurs in 0.4-1% of domestic cats, and prevalence appears to be increasing (Prahler et al., 2007; McCann et al., 2007; Reusch, 2010). In humans, diabetes is classified as type 1 or type 2 based on etiopathogenesis; type 1 diabetes is characterized by absolute insulin deficiency and immune-mediated destruction of pancreatic β-cells, whereas type 2 involves a combination of insulin resistance and potentially reversible β-cell dysfunction. The most common form of diabetes in cats resembles human type 2 diabetes in several respects. Specifically, emergence of clinical diabetes in cats depends on the balance between insulin sensitivity and β-cell function (Nelson et al., 1999; Reusch, 2010; Hoenig, 2012); as well, for both human type 2 and feline diabetes, obesity is a predisposing factor (Panciera et al., 1990; Buse et al., 2003).

Obesity leads to insulin resistance in cats and humans (Buse et al., 2003; Hoenig et al., 2007). In one study, insulin sensitivity in cats was estimated to decrease by 30% for each 1-kg increase in body weight (Hoenig et al., 2007). Impaired insulin sensitivity, from obesity or other causes, is accompanied by an increase in insulin secretion; this is referred to as compensatory hyperinsulinemia, and is an allostatic response that initially allows maintenance of normal blood glucose concentrations (Buse et al., 2003; Hoenig et al., 2011). In a subset of insulin-resistant cats, however, β-cells are ultimately unable to sustain this high level of insulin production.
When insulin secretion becomes inadequate to overcome insulin resistance, overt diabetes develops (Hoenig et al., 2000).

Current treatment options for feline diabetes are limited to insulin and the sulfonylureas. Although insulin is effective in many cases, it must be given by injection and carries the risk of hypoglycemia, both of which can be significant concerns for owners (Niessen et al., 2010). Sulfonylureas are insulin secretagogues that inhibit an ATP-dependent K⁺ channel on β-cell membranes. Glipizide, a member of this drug class, has been used in diabetic cats; unfortunately, long-term treatment with glipizide promotes amyloid deposition in pancreatic islets, and does not enhance potential for β-cell recovery (Hoenig et al., 2000). A larger number of oral treatment choices for feline diabetes, particularly for owners unable or unwilling to attempt insulin therapy, would be beneficial.

The thiazolidinediones (TZDs) are oral insulin sensitizers marketed for use in human type 2 diabetes. These drugs are agonists of the peroxisome proliferator activated receptor gamma (PPARγ), a nuclear transcription factor that is a key regulator of glucose metabolism, lipid metabolism, and adipogenesis (Lehmann et al., 1995; Tontonoz & Spiegelman, 2008). Treatment of humans and rodents with TZDs increases whole-body insulin sensitivity and promotes uptake and storage of circulating lipids by adipocytes (Yki-Järvinen, 2004); in type 2 diabetics, the result is improved glycemic control, reduced plasma lipid concentrations, and redistribution of lipids from sites of ectopic accumulation (muscle and liver) to adipose tissue (Aronoff et al., 2000; Yki-Järvinen, 2004; Rasouli et al., 2005; Bajaj et al., 2010). Unlike sulfonylureas, TZDs have beneficial long-term effects on pancreatic β-cells, increasing β-cell sensitivity to glucose in human diabetics (Gastaldelli et al., 2007) and preserving islet structure in diabetic rodents (Diani
et al, 2004). Also, as insulin sensitizers, they are associated with a low risk of hypoglycemia when used as monotherapy (DeFronzo, 2010).

Because the TZDs are effective for type 2 diabetes in humans, they may prove useful for treatment of diabetes in cats. An experimental TZD, darglitazone, increased glucose and non-esterified fatty acid (NEFA) clearance during glucose challenge in cats with obesity-induced insulin resistance (Hoenig & Ferguson, 2003). Darglitazone did not complete clinical development in either humans or animals; however, the TZD pioglitazone was approved for human use in 1999 and has remained commercially available (US FDA, 1999, Approval). The purpose of this study was to evaluate the effects of pioglitazone in obese, insulin-resistant cats, in order to assess its potential for future use in feline diabetes mellitus. We hypothesized that pioglitazone would increase insulin sensitivity and lead to enhanced glucose and lipid disposal in obese cats.

4.3 Materials and Methods

Animals

Twelve Domestic Shorthair cats, six neutered males and six spayed females, 5-7 years of age, were used for this study. At the beginning of the study, the cats weighed 5.4-9.8 kg (median 6.2 kg), and were classified as obese based on gain of ≥50% of their adult lean body weight (median percent gain 99%; range 57-107%). Obesity had originally been induced by ad libitum feeding, and all cats had been obese for >1 year. Cats were individually housed at the University of Illinois veterinary medical animal care facility, with free access to water, and were fed a commercial dry maintenance diet (Purina ProPlan Chicken and Rice™, Nestlé Purina, St. Louis, MO, USA) once a day. Food intake was recorded daily and adjusted to maintain body weight,
which was recorded weekly. All study procedures were approved by the university's Institutional Animal Care and Use Committee and conducted in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

_Treatments_

Cats were stratified by sex and percent gain of adult lean body weight, and randomly allocated to three treatment groups, either by drawing numbers from a box or by using a random number generator. The same randomization method was used within each stratum. Each group contained 2 males and 2 females, and there were no statistically significant differences in median percent gain or body weight between groups. A treatment sequence was assigned to each group using a random number generator. Treatments consisted of seven weeks of placebo, 1 mg/kg oral pioglitazone, or 3 mg/kg oral pioglitazone, with each treatment followed by a seven-week washout period. All cats received all treatments, in a 3-way crossover design. Treatment sequences are shown in Table 4.1.

During each dosing period, pioglitazone (Actos™ 15 mg tablets, Takeda Pharmaceuticals America, Inc., Deerfield, IL, USA) or placebo was administered once daily, in the morning, with a small of canned food (Hill’s a/d™, Hill’s Pet Nutrition, Topeka, KS, USA, and Purina DM™, Nestlé Purina, St. Louis, MO, USA). Actos™ tablets were divided using a pill cutter, weighed, and placed in gelatin capsules prior to administration. Placebo capsules contained ~186 mg powdered lactose (D-lactose monohydrate, USP-NF, Fisher Scientific, Pittsburgh, PA, USA), a quantity equivalent to the approximate amount of lactose in the greatest
number of pioglitazone tablets to be received by any cat during any period of the study. Feeding of the daily ration was conducted without regard to the time of dosing.

*Physical examination, laboratory testing, and echocardiography*

Before each dosing period and during weeks 6-7 of dosing, a physical examination, complete blood count (CBC), biochemistry panel, and echocardiogram were performed on each cat. Total thyroid hormone (T4) was also measured prior to the first dosing period. Echocardiographic monitoring was elected based on data from preclinical toxicity studies of pioglitazone, in which hydrothorax and biventricular hypertrophy were noted after chronic dosing in some laboratory species (US FDA, 1999, Part 6).

All laboratory testing was performed through the Clinical Pathology Laboratory at the University of Illinois College of Veterinary Medicine. Echocardiograms were performed by a board-certified veterinary radiologist at the University of Illinois College of Veterinary Medicine and included 2-dimensional screening for thoracic effusion or obvious atrial enlargement, as well as M-mode measurements of left ventricular posterior wall thickness in diastole (LVPWd), left ventricular internal diameter in systole and diastole (LVIDs, LVIDd), interventricular septal thickness in systole and diastole (IVSs, IVSd), % fractional shortening (FS), and left atrial/aortic (LA/Ao) ratio. Respiratory rate, heart rate, and results of thoracic auscultation were recorded daily prior to dosing, and these data were evaluated visually during the dosing periods to identify trends in individual cats.
Indirect calorimetry

For detection of any species-specific effects of pioglitazone on substrate metabolism or energy expenditure, indirect calorimetry was performed before each dosing period and during the 6th week of dosing. Cats were fasted overnight and placed in 50 cm x 50 cm x 41 cm Plexiglass chambers connected to an open-circuit calorimetry system (Oxymax, Columbus Instruments, Columbus, OH). The cats remained in the chambers for 8 hours, with free access to water throughout the experiment. System flow rate was 5 L/min, settle time was 60 s, and measure time for each chamber was 30 s. Calibration was performed daily using known calibration gas standards, and room temperature was maintained at 25 ± 1° C. Basal heat production and respiratory exchange ratio were calculated according to the following equations:

\[
RER = \frac{\text{liters CO}_2 \text{ produced}}{\text{liters O}_2 \text{ consumed}}
\]

Heat production (kcal) = 3.82 x liters O2 consumed +1.15 x liters CO2 produced

Heat production per metabolic body size (HMBS) = heat production (kcal/kg)/(body weight)^0.75

Intravenous glucose tolerance testing

Intravenous glucose tolerance tests (IVGTTs) were performed immediately before each dosing period, and during the 7th week of dosing. At least 18 hours prior to IVGTT, cats were sedated with tiletamine/zolazepam (Telazol™, Fort Dodge, IA, USA; 2-4 mg/kg intramuscularly based upon tiletamine) to allow placement of jugular catheters (Intracath, Becton-Dickinson, Franklin Lakes, NJ, USA). Catheter patency was maintained by flushing with sterile 0.38%
sodium citrate. After a 24-hour fast, a bolus of sterile 50% dextrose (0.8 g/kg) was administered intravenously, and samples were withdrawn from the jugular catheters at -5, 0 (immediately prior to dextrose administration), 5, 10, 15, 30, 45, 60, 90, 120, and 180 minutes. Samples were placed in EDTA tubes on ice until centrifugation (1500 g for 10 minutes). Plasma was separated, aliquoted for measurement of glucose, insulin, NEFAs, and the adipocytokines adiponectin and leptin (time 0 only), and stored at –20 C.

**Pioglitazone concentrations**

During each post-treatment IVGTT, selected samples from dosed animals were aliquoted for measurement of pioglitazone concentration. On the same day, three additional samples were collected, such that plasma was available for pioglitazone measurement at 0h (predose), 2h, 3h, 4h, 5h, 8h, and 11h after drug administration. The additional samples were centrifuged and stored as described above.

**Assays**

Glucose was measured by a colorimetric glucose oxidase method (Glucose (Trinder) Assay, Genzyme Diagnostics, Charlottetown, PEI) and insulin by a porcine insulin radioimmunoassay with human insulin standards (Porcine Insulin RIA, Millipore, Billerica, MA, USA). NEFAs were quantitated using an enzymatic colorimetric kit (NEFA-HR2, Wako Diagnostics, Richmond, PA, USA.) Adiponectin was measured by ELISA (Human Adiponectin ELISA Kit, B-Bridge International, Inc., Sunnyvale, CA, USA), and leptin was measured by a commercial radioimmunoassay (Multispecies Leptin RIA kit, Linco, St. Charles, MO, USA). All samples were assayed in duplicate. The adiponectin and leptin assays had been previously
validated in our laboratory for use in cats (Hoenig & Ferguson, 2002; Hoenig et al., 2007), and all samples were processed in the same assay. For the insulin assay, the standard curve for diluted feline samples was parallel to the curve for human insulin standards. Recovery of insulin from spiked samples in blank feline plasma was 103% at low and 94% at high concentrations. Inter-assay and intra-assay coefficients of variation were 6.3% and 11.9%, respectively. Pioglitazone concentrations were determined by high-performance liquid chromatography (HPLC), using a previously validated procedure (Clark et al., 2012).

Data analysis

Baseline values for glucose, insulin, and NEFAs were calculated by taking the average of the measurements at -5 and 0 minutes of the IVGTT. AUC\textsubscript{0-180} for glucose and insulin concentrations, AUC\textsubscript{0-90} and AUC\textsubscript{0-180} for NEFA concentrations and percent NEFA suppression, and coefficient of glucose disappearance (k) were calculated for each IVGTT using statistical software (GraphPad Prism Version 5.00 for Windows, Graph Pad Software, San Diego, CA, USA, or the R software package (Pinheiro et al., 2012)). The AUC\textsubscript{0-180} for glucose, NEFAs and insulin were calculated both in absolute terms and as delta AUC (AUC above or below baseline values), and percent NEFA suppression was calculated at each time point using the following formula: \(\text{% suppression} = 100 - (\text{NEFA}_0/\text{NEFA}_t) \times 100\), where NEFA\textsubscript{0} is NEFA concentration at baseline and NEFA\textsubscript{t} is NEFA concentration at time \(t\) after baseline. Coefficient of glucose disappearance was calculated using the one-phase decay equation \(C = (C_0 - \text{Plateau}) \times e^{(-\frac{k}{t})} + \text{Plateau}\), where \(C_0\) is extrapolated maximum glucose concentration at time 0, \(t\) is time, and Plateau is the value of \(C\) at infinite time.
Post-treatment body weights were obtained prior to the IVGTT (during week 6 of dosing), and average daily food intake was calculated for each treatment period from day 1 of dosing to the day prior to indirect calorimetry (5-5.5 weeks of treatment) to avoid days when cats were fasted for assessments. RER and heat production were calculated for each cat by taking the average of all measurements obtained over a 2-hour period (hours 4-6 of the experiment). $C_{\text{max}}$, AUC$_{0-\text{inf}}$, terminal elimination half-life ($t_{1/2}$), oral clearance (Cl/F), and oral volume of distribution ($V_z/F$) of pioglitazone for each cat were estimated from the concentration-time data using commercial software (Phoenix WinNonlin 6.1, Pharsight Corporation, Cary, NC, USA).

The changes in basal and integrated glucose, NEFA, and insulin concentrations, integrated percent NEFA suppression, coefficient of glucose disappearance, body weight, adipocytokines, calorimetry data, clinical pathology values, and echocardiographic measurements were compared among treatments using linear mixed-effects modeling with logarithmic transformation of the data. The factor “stage” was created to classify data as pre- or post-treatment for each period, and interactions of stage with treatment, period, and sequence were included in the regression model as fixed effects; when stage*period and stage*sequence were not significant, they were removed from the final model. If a significant stage*sequence interaction was identified, its potential interference with the estimate of treatment effects was further evaluated using stage*treatment*sequence. Variables with only one measure per period (pharmacokinetic parameters, food consumption, frequency of adverse events) were analyzed in a similar fashion, but fixed effects were treatment, period and sequence rather than interactions of these factors with the factor “stage”. Cat and treatment within cat were included in the regression models as random effects.
Modeling was implemented in the R statistical software package (Pinheiro et al., 2012), with P-values <0.05 considered significant. No correction for multiple comparisons was applied during analysis of clinicopathologic or echocardiographic data, since avoidance of Type II error (false negatives) rather than Type I error (false positives) was considered preferable in this circumstance. Placebo was used as a reference for contrasts (except in the case of pharmacokinetic parameters); therefore, magnitudes of all reported changes with pioglitazone administration take into account the corresponding change with placebo administration.

Insulin sensitivity, defined as the response of both glucose and NEFAs to endogenous insulin secreted during IVGTT, was quantified and compared among treatment groups as follows:

Dynamic glucose-insulin data were analyzed by means of the minimal model (MM) of glucose disappearance (Bergman et al., 1979), which is described by the following differential equations

\[
\frac{dG(t)}{dt} = -(S_G + X(t))G(t) + S_G G_b ; \quad G(0) = G_b + \frac{D_G}{V_G}
\]

\[
\frac{dX(t)}{dt} = -P_2[X(t) - S_I(I(t) - I_b)] ; \quad X(0) = 0.
\]

Plasma glucose concentration, \(G(t)\), is the predicted model output that corresponds to an impulsive glucose administration of a known dose \(D_G\) and with a given insulin concentration time course, \(I(t)\), viewed as known model input. Insulin acts on glucose disappearance through the delayed insulin action term \(X(t)\). Model parameters \(G_b\) and \(I_b\) are the measured basal glucose and insulin concentrations, respectively, and the remaining model parameters are estimated: \(S_G\) (fractional glucose clearance at basal insulin); \(S_I\) (insulin sensitivity of fractional glucose
disappearance); \( P_2 \) (rate constant of time lag between supra-basal changes in insulin concentrations and insulin action, \( X(t) \)); and \( V_G \) (apparent distribution volume).

Dynamic NEFA-insulin data were analyzed by a reparameterized version of a previously proposed model of NEFA kinetics (Thomaseth & Pavan, 2005). The dynamic model equations resemble those of the MM, in particular the dynamics of insulin action, which in this case suppresses part of the circulating NEFAs. The model is defined as:

\[
\frac{dN(t)}{dt} = -k_N(N(t) - N_{Id} \cdot |1 - X_N(t)|^+ - N_{Ii}) \; ; \; N(0) = N_{Id} + N_{Ii}
\]

\[
\frac{dX_N(t)}{dt} = -P_{2N}[X_N(t) - S_{IN}(I(t) - I_b)] \; ; \; X_N(0) = 0
\]

where \( N(t) \) represents plasma NEFA concentration and \( X_N(t) \), by analogy with insulin action \( X(t) \) in the MM, insulin action on NEFA suppression. The time-lag between supra basal changes in plasma insulin and NEFA suppression is characterized by the rate constant \( P_{2N} \). \( N_{Id} \) and \( N_{Ii} \) represent insulin-dependent and insulin-independent NEFA concentrations at basal, respectively. The term \( |1 - X_N(t)|^+ \) concisely describes the suppression of NEFA as a piece-wise linear function of insulin action \( X_N(t) \). (The notation \( |x|^+ \) represents the positive part of \( x \), i.e. \( |x|^+ = x \) if \( x > 0 \), and 0 otherwise). Parameter \( S_{IN} \) determines the slope of the suppression line, \( 1 - X_N(t) \), as long as \( X_N(t) < 1 \), as a function of deviations of insulin concentration from basal. The parameter \( S_{IN} \) bears therefore the interpretation of sensitivity of fractional NEFA inhibition to changes in plasma insulin around basal. Unlike other proposed models of NEFA kinetics (Periwal et al., 2008), the present model is able to cope with insulin concentrations below basal by predicting an increase in NEFA concentrations. The present model defines therefore a finite,
non-zero insulin sensitivity of NEFA suppression around basal insulin and not only for positive increases as in Periwal et al (2008).

Because the final NEFA concentration at 180 minutes was considerably higher than the concentration at the previous sample at time 120 min, the data considered for model fitting were restricted to the interval 0 to 120 minutes. Regarding the identifiability of model parameters, the sampling during the initial NEFA decline was not sufficiently dense to allow the assessment of NEFA disappearance rate, under the hypothesis of complete inhibition of the insulin-dependent NEFA production. Moreover, the oscillatory pattern in the data during the first 15 minutes did not corroborate the expected first order elimination rate of NEFA under complete inhibition. For these reasons, it was assumed that NEFA clearance occurs with a half-life of 3 min (hence parameter $k_N$ was set to 0.231 min$^{-1}$), and the 5 and 10 minute sample values were excluded from the final analysis.

The MM parameters ($S_G$, $S_I$, $P_2$, and $V_G$), and the parameters of the NEFA kinetics model ($N_{Id}$, $N_{Ii}$, $S_{IN}$, $P_{2N}$) were estimated by fitting the two models to the corresponding glucose and NEFA concentrations, using in both cases the same measured insulin profile as model input. Parameter estimation was carried out using the statistical nonlinear mixed effects (NLME) modeling approach. From a population pharmacokinetic point of view, parameters were assumed log-normally distributed among the experiments performed in different cats or repeated in the same cats under different experimental conditions. The likelihood ratio test and Akaike's Information Criterion (AIC) were used to test significance of random effects to be included in the NLME model, whereas significance levels of fixed-effects parameter estimates was used as primary criterion for inclusion of a covariate into the regression model. The significance
level was set at 0.05.

The effect of treatment on after - before differences in insulin sensitivity of glucose disappearance, $S_I$, and in insulin sensitivity of NEFA suppression, $SI_N$, was explicitly tested by including into the fixed effects regression model the interaction term stage * treatment. Stage*period and stage*sequence were also included, but were retained only when the corresponding parameter estimates were significant. In the case of marginal significance, the likelihood ratio test and AIC were used as the primary criteria for decision between models including the interaction terms and those including only sequence and/or period in addition to stage*treatment. All model identifications and parameter estimations were carried out using the nonlinear mixed effects package (Pinheiro et al., 2012). Mathematical model equations and simulation code were generated using the modeling software tool PANSYM (Thomaseth, 2003).

4.4 Results

Clinical observations, clinicopathologic data, and echocardiography

Throughout the study, no significant abnormalities were detected on physical examinations. No differences were found among treatments in clinical pathology or echocardiographic measurements, with the exception of decreases in triglycerides (35% decrease; $P=0.047$), cholesterol (13% decrease; $P=0.004$), eosinophils (57% decrease; $P=0.026$), and phosphorus (8% decrease; $P=0.018$) with 3 mg/kg pioglitazone, and a decrease in cholesterol (9% decrease, $P=0.034$) with 1 mg/kg pioglitazone. Values for these variables remained above the lower limit of the reference range in all cats. Decreases in serum alkaline phosphatase (ALP) (9% decrease; $P=0.024$) and IVSs (12% decrease; $P=0.014$) were also noted after 3 mg/kg pioglitazone administration; however, for the changes in these variables, there was a significant
sequence effect, and because this effect was unequal among treatments (as indicated by the presence of a significant stage*treatment*sequence interaction), the contribution of pioglitazone administration could not be reliably determined.

One or more episodes of vomiting occurred in 10/12 cats during the 8 months of the study. Vomiting was rarely temporally associated with drug administration (within 1 hour of dosing in <5% of occurrences), and was not associated with other clinical signs of illness. The frequency was not significantly different for either dosage of drug vs. placebo (P=0.36 and P=0.76 for 1 and 3 mg/kg, respectively). One cat died during sedation for catheter placement at the end of the last study period; necropsy and histopathology revealed myocardial fiber disarray characteristic of hypertrophic cardiomyopathy.

Glucose and NEFA disposal

Pre- and post-treatment glucose, NEFA, and insulin concentrations vs. time during glucose tolerance testing are shown in Figures 4.1, 4.2, and 4.3, respectively. Mean pre- and post-treatment fasting glucose, insulin and NEFA concentrations are shown in Table 4.2, and AUC\(_{0-180}\) for glucose, insulin, and NEFAs, along with coefficient of glucose disappearance (k-value), during the glucose tolerance test are shown in Table 4.3.

There were no differences among treatments with respect to the change in fasting glucose or NEFA concentrations, absolute glucose AUC\(_{0-180}\), delta (above basal) glucose AUC\(_{0-180}\), or coefficient of glucose disappearance (k-value). However, there was a significant decrease in insulin AUC\(_{0-180}\) with 3 mg/kg pioglitazone (34% decrease; P=0.003). This difference was still present when insulin AUC\(_{0-180}\) was expressed as delta (above basal) AUC\(_{0-180}\) (44% decrease;
Fasting insulin was also lower after 3 mg/kg pioglitazone, but the P-value for this change was at the borderline of significance (39% decrease; P=0.052).

NEFA AUC\textsubscript{0-180} decreased with 1 mg/kg pioglitazone (19% decrease; P=0.021) but not with 3 mg/kg pioglitazone (P=0.29). When NEFA AUC\textsubscript{0-180} was expressed as delta (below basal) NEFA concentration, the decrease with 1 mg/kg treatment was no longer significant (P=0.12). This suggested that it was dependent on trends in the baseline values (although, as mentioned above, changes in these fasting baseline values were not significantly different for either dose of drug vs. placebo). Plotting the NEFA concentrations as percent suppression from baseline revealed a subjectively greater initial suppression, and an earlier, more precipitous rebound from suppression, as the dosage increased (Figure 4.4). The steeper rebound for pioglitazone vs. placebo was also evident in the absolute NEFA concentration vs. time profiles (Figure 4.3).

Because of the pattern observed in the NEFA profiles, AUC for percent NEFA suppression was calculated both as AUC\textsubscript{0-180} (total NEFA suppression) and as AUC\textsubscript{0-90} (initial NEFA suppression, excluding the rebound phase) (Table 4.3). AUC\textsubscript{0-180} for percent NEFA suppression increased by 33% (P=0.036) with 1 mg/kg pioglitazone, but the change with the 3 mg/kg dosage was not significant (P=0.26). In the case of AUC\textsubscript{0-90} for percent NEFA suppression, however, there was an increase with both 1 and 3 mg/kg pioglitazone (43% increase, P=0.025, and 36% increase, P=0.048, respectively). AUC\textsubscript{0-90} was also calculated for absolute NEFA concentrations (Table 4.4). The results for NEFA AUC\textsubscript{0-90} were similar to those for NEFA AUC\textsubscript{0-180} for 1 mg/kg pioglitazone (23% decrease; P=0.031); for 3 mg/kg pioglitazone, the change in NEFA AUC\textsubscript{0-90} was at the borderline of significance (21% decrease, P=0.053).
For all calculations involving AUC for percent NEFA suppression, it was necessary to eliminate two profiles with an average suprabasal NEFA increment from analysis, as they yielded negative AUCs that were not amenable to log transformation. Because one of these profiles occurred after placebo, and one after 1 mg/kg pioglitazone, their exclusion had the potential to artificially improve the measures of percent NEFA suppression for both placebo and the 1 mg/kg dosage. Therefore, although the validity of the changes in percent NEFA suppression with 1 mg/kg pioglitazone vs. placebo is questionable, the increase in AUC<sub>0-90</sub> for percent NEFA suppression with 3 mg/kg pioglitazone vs. placebo may actually have been greater than reported.

**Insulin sensitivity**

Insulin sensitivity of glucose disposal, \( S_I \), increased by 141% after treatment with 3 mg/kg pioglitazone (P=0.0014). Administration of 1 mg/kg pioglitazone increased \( S_I \) by 46%, but this difference was not statistically significant (P=0.15). Insulin sensitivity of NEFA suppression, \( S_{IN} \), increased by 108% with 3 mg/kg pioglitazone (P=0.014). The change with 1 mg/kg pioglitazone was not significant (35% increase; P=0.37). Average best fit of the NEFA kinetic model to measured NEFA concentrations is shown in Figure 4.5.

**Adipokines**

Mean pre- and post-treatment values for adiponectin and leptin are shown in Table 4.2. Adiponectin concentration increased by 127% (P=0.0024) after 1 mg/kg pioglitazone treatment, and by 289% (P<0.0001) after 3 mg/kg pioglitazone treatment. There was no effect of pioglitazone on plasma leptin concentration.
Energy expenditure, body weight, food intake, and substrate metabolism

Pre- and post-treatment values for body weight, average daily food intake, RER, heat production (kcal/h), and heat production per metabolic body size (kcal/h/kg) are shown in Table 4.4. There was a statistically significant but very small decrease in mean RER after treatment with both 1 and 3 mg/kg pioglitazone (1.5% decrease, P=0.014, and 1.3% decrease, P=0.036, respectively). There were no differences among treatments for changes in the other variables.

Plasma pioglitazone concentrations

Plasma concentration-time profiles for 1 and 3 mg/kg administration of oral pioglitazone are shown in Figure 4.6. Mean ± SD C_{max}, AUC_{0-inf}, and terminal elimination half-life estimated by noncompartmental analysis of concentration-time profiles are displayed in Table 4.5.

Mean C_{max} and AUC_{0-inf} for pioglitazone were greater after 3 mg/kg than after 1 mg/kg administration (P≤0.001), as expected, although the increases were less than proportional. There was no difference in mean terminal elimination half-life between dosages (P=0.997). However, mean oral clearance (Cl/F) and apparent volume of distribution (V_{z}/F) were higher after 3 mg/kg than after 1 mg/kg administration (for Cl/F, mean ± SD 297 ± 111 vs. 155 ± 58 mL/kg/h, P<0.001; for V_{z}/F, 1.5 ± 0.51 vs. 0.78 ± 0.25 L/kg, P=0.003).

Trough pioglitazone concentration (pre-dose time point; 22-23h after the last dose) was below the LLOQ of the assay for 10/11 1 mg/kg administrations and for 7/11 3 mg/kg administrations. The maximum trough plasma concentration for any cat was 42 ng/mL for 1
mg/kg pioglitazone and 68 ng/mL for 3 mg/kg pioglitazone, indicating minimal accumulation of parent drug after chronic administration.

4.5 Discussion

The insulin-sensitizing properties of pioglitazone have been well described in obese and type 2 diabetic humans, and results of this placebo-controlled study demonstrate that pioglitazone, at 3 mg/kg, increases insulin sensitivity in obese cats. Improved insulin sensitivity was suggested by the significant decrease in AUC for insulin and the lack of an increase in AUC for glucose or NEFAs during IVGTT in view of lower insulin concentrations. Additionally, it was confirmed for both glucose and NEFA concentrations using an approach based on the minimal model of glucose kinetics. The insulin sensitivity index ($S_I$) generated by the minimal model reflects the actions of insulin to stimulate peripheral glucose uptake and suppress hepatic glucose production, whereas its counterpart ($S_{IN}$) in the corresponding NEFA model describes the potency of insulin to promote fatty acid uptake and inhibit peripheral lipolysis. The dual improvement in these indices in the obese cats is consistent with findings in other species that pioglitazone amplifies insulin signaling in multiple organs, including muscle, adipose tissue, and the liver (Sugiyama et al., 1990; Hofmann & Colca, 1992; Bajaj et al., 2004; Yki-Järvinen, 2004).

The improvement in insulin sensitivity with pioglitazone was less than that observed with the experimental TZD darglitazone (Hoenig & Ferguson, 2003), which can be explained by the difference in potency between these two agents (Day et al., 1999; Aleo et al., 2003). However, the impact of insulin sensitization with pioglitazone was still evident from several changes that occurred during IVGTT. First, the decrease in insulin AUC represents the β-cell response to
increased insulin sensitivity, and reflects reduced secretory demand and lessening of the stimulus for compensatory hyperinsulinemia. Alleviation of compensatory hyperinsulinemia was also present after darglitazone administration in obese cats (Hoenig & Ferguson, 2003), and is a predictable outcome of TZD administration in obese, insulin-resistant humans and rodents (Ikeda et al., 1990; Nolan et al., 1994; Powell et al., 2012). Because chronic β-cell overstimulation is associated with increased amyloid deposition in diabetic cats (Hoenig et al., 2000), the lower secretory demand posed by pioglitazone may offer benefits in terms of β-cell function and survival. In transgenic mice expressing human islet amyloid polypeptide, both the TZD rosiglitazone and another insulin sensitizer, metformin, decreased the severity of amyloid deposition in conjunction with limiting β-cell apoptosis (Hull et al., 2005).

Second, 3 mg/kg pioglitazone enhanced NEFA suppression during the first 90 minutes of IVGTT. Potential mechanisms for this change include decreased NEFA release (due to greater inhibition of lipolysis), increased NEFA uptake, or some combination of the two. In obese Zucker rats, in which the actions of TZDs on NEFA metabolism have been studied in detail, TZDs enhance both lipolytic inhibition and NEFA uptake under insulin-stimulated conditions. This leads to a net flux of NEFAs into peripheral adipose depots and a corresponding depletion of ectopic lipid from other tissues, as the majority of the NEFA uptake is into adipocytes (Oakes et al., 2001). Consequently, TZD treatment in Zucker rats is associated with a decrease in the triglyceride content of muscle, liver, and pancreatic β-cells (Shimabukuro et al., 1998; Hockings et al., 2003). Similar effects in terms of both lipolytic inhibition and muscle and liver triglyceride content of pioglitazone-treated humans (Ravikumar et al., 2008; Gastaldelli et al., 2009; Bajaj et al., 2010). Thus, it might be postulated that preferential adipose tissue NEFA
uptake and lipolytic inhibition were components of NEFA suppression in the obese cats, although this would require further study for confirmation.

An interesting feature of the post-pioglitazone NEFA profiles in the obese cats was the brisk suprabasal rebound that followed initial suppression. In people, this has been attributed to lipolytic hormone release secondary to rapid glucose clearance, and is faster in lean than in obese individuals (Bolinger et al., 1962; Prando, Cordera, DeMicheli et al., 1978; Prando, Cordera, Odetti et al., 1978). A similar phenomenon has been described after pharmacological doses of glucose administered orally, and the magnitude of the NEFA rebound in one study was positively correlated with insulin sensitivity (Fernandes et al., 2012). The subjectively steeper rebound with pioglitazone in the cats, therefore, appears compatible with a return to a more insulin-sensitive state, and may actually reflect a biologically significant increase in glucose clearance. Based on the lack of a concurrent change in glucose AUC or k-value, this possible effect of pioglitazone on glucose clearance was more subtle than its effects on NEFA metabolism, which may seem surprising considering the slightly greater change in $S_I$ than in $S_{IN}$. A potential explanation is that peripheral lipolysis is inherently more responsive to insulin than is glucose uptake (Zierler & Rabinowitz, 1964; Yki-Järvinen et al., 1987); thus, a comparable increase in the insulin sensitivity of both processes will be manifest to a greater degree in NEFA than in glucose dynamics.

In general, the effects of TZDs on glucose and NEFAs are more pronounced in diabetic than in obese humans and rodents, and even among non-diabetic humans with impaired glucose tolerance, the glycemic effects of TZDs are greatest in those with the highest initial glucose concentrations (Nolan et al., 1994; Frias et al., 2000; Powell et al., 2012). This is distinct from results observed with administration of sulfonylureas to non-diabetic subjects (Unger &
Madison, 1958; Groop et al., 1987), and is a consequence of the fact that TZDs improve the tissue response to insulin without disrupting endogenous glucose-insulin feedback. Thus, given its effects on insulin sensitivity in obese cats, pioglitazone may favorably affect glycemic control in diabetic cats. Also, in light of its effect on NEFA suppression in obese cats, it may improve NEFA metabolism in diabetic cats and reduce complications such as hepatic lipid accumulation. One caveat with regard to these hypotheses, however, is that TZDs are clinically ineffective in the absence of insulin (Ikeda et al., 1990; Day et al., 1999). Therefore, although the insulin-sensitizing actions of pioglitazone in the obese cats are encouraging in terms of its therapeutic potential, effects in diabetic cats remain speculative and would depend on insulin secretory capacity, which has been shown to be low in some diabetic cats (Crenshaw & Peterson, 1996; Nelson et al., 1999).

The changes in serum lipids that occurred with pioglitazone in the obese cats are comparable in some respects to those that occur in pioglitazone-treated humans. Insulin resistance in humans, rodents, and cats is associated with dyslipidemia characterized by an increased mass of triglyceride (TG) in very low-density lipoproteins (VLDL), coupled with decreases in low-density lipoprotein (LDL) particle size and high-density lipoprotein (HDL) particle size and concentration (Ginsberg et al., 2005; Jordan et al., 2008). In diabetic humans and Wistar fatty rats, pioglitazone lowers triglyceride concentrations by increasing the clearance of VLDL-TG from circulation; this is thought to occur through an increase in the activity of lipoprotein lipase (LPL), an insulin-regulated enzyme that mediates VLDL-TG hydrolysis (Kazumi et al., 1996; Nagashima et al., 2005). A similar mechanism may have been responsible for the decrease in triglycerides in the obese cats.
With regard to cholesterol, the effects of pioglitazone in humans differ slightly from those observed in the obese cats; pioglitazone increases HDL cholesterol in obese or diabetic humans, but generally does not change total cholesterol concentration (Nagashima et al., 2005; Szapary et al., 2006; Powell et al., 2012). This difference between humans and cats may relate to the fact that 1) cats, unlike humans, carry most of their cholesterol in HDL, and 2) activity of cholesteryl ester transfer protein (CETP), which catalyzes the exchange of triglycerides and cholesterol between HDL and other lipoproteins, appears to be very low in cats (Watson, 1996). Rodents share both of these characteristics of cholesterol metabolism with cats (Hogarth et al., 2003), and pioglitazone does reduce total cholesterol in obese and diabetic rats (Mizushige et al., 2002; Wallis et al., 2004; Hirasawa et al., 2008). Since islet cholesterol homeostasis is now recognized as an important regulatory factor in glucose-stimulated insulin secretion (Hao et al., 2007; Kruit et al., 2010), and since hypercholesterolemia has been identified as a negative predictor of diabetic remission in cats (Zini et al., 2010), it is possible that the cholesterol-lowering effect of pioglitazone, as observed in the obese cats, would ultimately impart clinical benefit in diabetic cats. Furthermore, the effects of pioglitazone on both triglycerides and cholesterol, as well as its influence on NEFA suppression, may eventually prove useful in cats with other dyslipidemias. Cats with idiopathic hepatic lipidosis have increased triglyceride content in all lipoproteins, and elevated NEFA concentrations due to excessive peripheral lipolysis (Pazak et al., 1998). Depending on the role of insulin resistance in feline hepatic lipidosis, pioglitazone administration could theoretically lead to improved lipid metabolism and mobilization of hepatic triglyceride in this disorder.

Although most other outcomes of pioglitazone administration were significant only at the 3 mg/kg dosage, even 1 mg/kg pioglitazone in the obese cats caused a robust increase in plasma
concentrations of adiponectin. Adiponectin is an adipose-derived cytokine with insulin-sensitizing, anti-apoptotic, and anti-inflammatory effects, and because its secretion decreases with increasing fat mass and insulin resistance, concentrations are abnormally low in obese and diabetic humans and cats (Yu et al., 2002; Hoenig, 2006; Hoenig et al., 2007; Turer & Scherer, 2012). The relevance of the increase in adiponectin with pioglitazone is twofold. First, it signifies activation of PPARγ, the molecular target of the TZDs; TZDs directly increase adiponectin expression through a PPARγ response element in the adiponectin promoter, and a dose-dependent increase in this cytokine is a reliable sequel of PPARγ agonist administration in humans and rodents (Maeda et al., 2001; Yu et al., 2002; Iwaki et al., 2003). Second, adiponectin is a possible mediator of the effects of pioglitazone on insulin sensitivity. It is required for the hepatic insulin-sensitizing actions of pioglitazone in genetically obese and hyperglycemic ob/ob mice (Kubota et al., 2006). As well, it appears to have insulin-sensitizing effects on muscle glucose uptake, hepatic glucose production, and lipoprotein metabolism in humans (Tschritter et al., 2003; Bajaj et al., 2004; Hulstrom et al., 2008).

In the cats of this study, pioglitazone produced no change in energy expenditure or mean body weight, and the small decreases in respiratory exchange ratio identified during indirect calorimetry are unlikely to be biologically significant. The effects of pioglitazone on energy expenditure were of interest because TZDs have been associated with weight gain in humans and rodents (Festuccia et al., 2008; Barnett, 2009). This has been ascribed to the adipogenic activities of PPARγ; however, as some investigators have pointed out (Ryan et al., 2011), an increase in adipogenesis will not produce weight gain unless it is supplemented by an increase in food intake or a decrease in energy expenditure. In normal rats, both a decrease in energy expenditure and an increase in food intake are seen with TZD administration, with the former
resulting from a drug-induced downregulation of sympathetic outflow and thyroid status (Festuccia et al., 2008). In contrast, pioglitazone does not alter energy expenditure in obese, diabetic humans, consistent with its effects in the obese cats (Smith et al., 2005). Also, the lack of a change in body weight in the cats, which were fed a fixed ration, is similar to reports in humans that weight gain did not occur when pioglitazone was administered with a portion controlled-diet (Martin et al., 2010).

In addition to weight gain, the adverse effects profile of pioglitazone in humans includes fluid accumulation (manifest as peripheral edema or, more rarely, congestive heart failure), mild, reversible decreases in red blood cell count, increased risk of distal limb fractures in postmenopausal women, and possible increased risk of bladder cancer with long-term use (>2 y) (Barnett et al., 2009; Loke et al., 2009; Lewis et al., 2011). Red blood cell decrement and fluid accumulation were also observed in mice, rats, dogs, and monkeys during preclinical testing (US FDA, 1999, Part 1; US FDA, 1999, Part 2; EMEA, 2004), and cardiac hypertrophy and thoracic effusion occurred in some of these laboratory animals at ≥5 times the human exposure and/or after durations of ≥1 year (US FDA, 1999, Part 1; US FDA, 1999, Part 6). These changes were considered to be a response to chronic volume overload rather than a result of direct cardiac toxicity (US FDA, 1999, Part 1; US FDA, 1999, Part 2).

In view of these potential adverse effects, cats in this study were monitored by physical examination, laboratory evaluation, and echocardiography. All cats appeared healthy prior to study entry based on these criteria; however, despite normal echocardiographic findings, one cat experienced cardiac arrest under sedation and had myofiber disarray typical of hypertrophic cardiomyopathy (HCM) at necropsy. This histologic abnormality is unlikely to have been caused by short-term pioglitazone administration, although exacerbation of pre-existing disease cannot
be ruled out. In the other obese cats, pioglitazone did not cause echocardiographic changes or clinical signs of toxicity when administered at 1 or 3 mg/kg for 6 weeks, and most laboratory parameters, including red blood cell count, were unaffected by pioglitazone at this dosage and duration. The clinical significance of the observed decreases in eosinophils and serum phosphorus is unclear, as such changes do not appear to have been reported with pioglitazone in other species, and neither variable fell below the reference range in any cat. Mild decreases in total white blood cell count have been observed with TZDs in some studies in obese humans, in association with decreases in other markers of systemic inflammation, but in these investigations, no reductions in specific leukocyte fractions were found to be responsible (Ghanim et al., 2006; Szapary et al., 2006).

The dosages of pioglitazone used in this study were selected based on a previous pharmacokinetic evaluation, and were designed to bracket the range of human therapeutic concentrations (Clark et al., 2012). Pioglitazone concentrations were measured after dosing in order to verify drug exposure, establish a concentration-response relationship, and detect changes in pharmacokinetics with chronic administration. $C_{\text{max}}$ and $AUC_{0-\text{inf}}$, as assessed by limited sampling, were similar to concentrations achieved previously in cats (Clark et al., 2012) and to concentrations considered therapeutic in humans (Eckland & Danhof, 2000; Kalliokoski et al., 2007), but increases in these parameters between dosages were less than proportional. Subproportional increases in $C_{\text{max}}$ and $AUC_{0-\text{inf}}$ have also been reported for pioglitazone in dogs, rodents, and diabetic humans, at dosages ranging from approximately 1 to 100 mg/kg (EMEA, 2004; US FDA, 1999, Part 1).

Either changes in bioavailability or changes in elimination with increasing dose may account for lack of dose proportionality. In the cats of this study, half-life was not different
between dosages, and was similar to values obtained previously after single oral and IV doses in cats (Clark et al., 2012; Chapter 3). However, estimates of oral clearance (Cl/F) and apparent volume of distribution (Vz/F) were approximately doubled after 3 mg/kg compared with 1 mg/kg administration. Half-life is affected by both clearance and volume of distribution, with greater clearance tending to decrease half-life and a greater volume of distribution tending to increase it. Although equivalent increases in Cl and Vz could theoretically have occurred with 3 mg/kg administration, leaving half-life unchanged, a more likely explanation for the changes in Cl/F and Vz/F is a relative decrease in oral bioavailability (F) at the higher dosage. This is also suggested by the lower Cmax with 3 mg/kg administration; a change in elimination rate alone would be likely to change AUC0-inf without necessarily altering Cmax. Comparison of the median doses and mean AUCs obtained here with those calculated in obese cats after a single IV dose yielded oral bioavailabilities of 84% and 47%, respectively, for 1 and 3 mg/kg pioglitazone. These estimates must be interpreted with caution due to the different study population and sampling schedule used to generate the IV data, and the use of mean and median parameters rather than individual values. Nonetheless, the apparent dose-dependent absorption characteristics of pioglitazone in cats, as suggested by the data presented here, may explain the fact that the absolute bioavailability calculated previously after a single 3 mg/kg oral dose (Clark et al., 2012) was lower than that reported with therapeutic dosages of pioglitazone in humans.

Although NEFA concentrations as a percentage of baseline appeared to increase after placebo dosing, no biological explanations for this observation are evident. The amount of powdered lactose in the placebo capsules was very small, and no reports of an effect of orally administered lactose on NEFA concentrations could be located in the literature. Regardless of
the cause, any change in the placebo group is not likely to have impacted study conclusions, as all comparisons were made using the placebo group as a reference.

In summary, oral pioglitazone (Actos™) significantly improved insulin sensitivity and lowered plasma cholesterol and triglyceride concentrations in obese, insulin-resistant cats, after 6 weeks of daily dosing at 3 mg/kg. No changes in energy expenditure were noted, and no overt clinical toxicity attributable to pioglitazone was evident in otherwise healthy obese cats at this dosage and duration. Based on these results, further investigation of pioglitazone in diabetic cats, or in cats with other lipid metabolic disorders, may be warranted.
4.6 Figures and Legends

Figure 4.1 Glucose concentrations (mean ± SEM) vs. time during IVGTT in obese cats (n=11-12) before and after 6 weeks of administration of placebo, 1 mg/kg oral pioglitazone, or 3 mg/kg oral pioglitazone.
Figure 4.2 Insulin concentrations (mean ± SEM) vs. time during IVGTT in obese cats (n=11-12) before and after 6 weeks of administration of placebo, 1 mg/kg oral pioglitazone, or 3 mg/kg oral pioglitazone.
**Figure 4.3** Non-esterified fatty acid (NEFA) concentrations (mean ± SEM) vs. time during IVGTT in obese cats (n=11-12) before and after 6 weeks of administration of placebo, 1 mg/kg oral pioglitazone, or 3 mg/kg oral pioglitazone.
Figure 4.4 Percent suppression of non-esterified fatty acid (NEFA) concentrations (mean ± SEM) vs. time during IVGTT in obese cats (n=11-12) before and after 6 weeks of administration of placebo, 1 mg/kg oral pioglitazone, or 3 mg/kg oral pioglitazone.
Figure 4.5 Average best fit of the non-esterified fatty acid (NEFA) kinetic model used to determine insulin sensitivity of NEFA suppression ($S_{IN}$) to NEFA concentrations measured during IVGTT in obese cats (n=11-12).
Figure 4.6 Pioglitazone concentrations (mean ± SEM) vs. time after 6 weeks of administration of 1 or 3 mg/kg oral pioglitazone (Actos\textsuperscript{TM}) to obese cats (n=11-12). Note logarithmic y-axis.
**Table 4.1** Treatment sequences used in the 3-way crossover design for assessing the effects of two dosages of oral pioglitazone vs. placebo in obese cats. Each dosing period was separated from the next by a 7-week washout period.

<table>
<thead>
<tr>
<th></th>
<th>Period 1</th>
<th>Period 2</th>
<th>Period 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence 1</td>
<td>Placebo</td>
<td>3 mg/kg</td>
<td>1 mg/kg</td>
</tr>
<tr>
<td>Sequence 2</td>
<td>1 mg/kg</td>
<td>Placebo</td>
<td>3 mg/kg</td>
</tr>
<tr>
<td>Sequence 3</td>
<td>3 mg/kg</td>
<td>1 mg/kg</td>
<td>Placebo</td>
</tr>
</tbody>
</table>

**Table 4.2.** Fasting glucose, insulin, NEFA, and adipocytokine concentrations in 12 obese cats before and after 6 weeks of treatment with placebo, 1 mg/kg pioglitazone, or 3 mg/kg pioglitazone. Values with non-normal distribution are expressed as median (IQR); normally distributed values are expressed as mean ± SE. *= significant change from pre-treatment value vs. change with placebo (P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>1 mg/kg pioglitazone</th>
<th>3 mg/kg pioglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>4.8 (4.3-5.2)</td>
<td>4.7 (4.3-5.7)</td>
<td>4.8 (4.3-5.2)</td>
</tr>
<tr>
<td>Post</td>
<td>4.3 (4.1-4.5)</td>
<td>4.2 (4.2-4.4)</td>
<td>4.3 (3.9-4.8)</td>
</tr>
<tr>
<td>Insulin, baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>66 (44-83)</td>
<td>76 (29-90)</td>
<td>47 (28-116)</td>
</tr>
<tr>
<td>Post</td>
<td>42 (32-81)</td>
<td>42 (23-81)</td>
<td>25 (11-49)</td>
</tr>
<tr>
<td>NEFA, baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mEq/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>0.70 (0.45-0.78)</td>
<td>0.66 (0.47-0.88)</td>
<td>0.65 (0.47-0.75)</td>
</tr>
<tr>
<td>Post</td>
<td>0.52 (0.42-0.87)</td>
<td>0.58 (0.54-0.74)</td>
<td>0.61 (0.56-0.70)</td>
</tr>
<tr>
<td>Adiponectin (μg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>1.7 ± 0.4</td>
<td>1.9 ± 0.4</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Post</td>
<td>2.3 ± 0.6</td>
<td>4.4 ± 0.9*</td>
<td>7.3 ± 1.3*</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>19 ± 3</td>
<td>16 ± 2</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>Post</td>
<td>17 ± 2</td>
<td>15 ± 2</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>
Table 4.3 AUC\(_{0-180}\) for glucose, insulin, NEFAs, and % NEFA suppression, and coefficient of glucose disappearance, during an IV glucose tolerance test in 12 obese cats before and after 6 weeks of treatment with placebo, 1 mg/kg pioglitazone, or 3 mg/kg pioglitazone. Values with non-normal distribution are expressed as median (IQR); normally distributed values are expressed as mean ± SE. *= significant change from pre-treatment value vs. change with placebo (\(P<0.05\)).

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>1 mg/kg pioglitazone</th>
<th>3 mg/kg pioglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose AUC (_{0-180})</strong>, (min*mol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>2.2 (1.9-2.5)</td>
<td>2.1 (2.0-2.2)</td>
<td>2.2 (1.8-2.5)</td>
</tr>
<tr>
<td>Post</td>
<td>2.1 (1.8-2.3)</td>
<td>1.8 (1.7-2.0)</td>
<td>1.8 (1.6-2.1)</td>
</tr>
<tr>
<td><strong>Insulin AUC (_{0-180})</strong>, (min*nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>27 (20-39)</td>
<td>25 (18-34)</td>
<td>26 (19-42)</td>
</tr>
<tr>
<td>Post</td>
<td>35 (24-47)</td>
<td>25 (20-34)</td>
<td>16 (13-34)*</td>
</tr>
<tr>
<td><strong>NEFA AUC (_{0-180})</strong>, (min*mEq/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>48 (42-59)</td>
<td>54 (48-64)</td>
<td>54 (46-58)</td>
</tr>
<tr>
<td>Post</td>
<td>51 (48-66)</td>
<td>50 (45-66)*</td>
<td>68 (49-82)</td>
</tr>
<tr>
<td><strong>NEFA AUC (_{0-90})</strong>, (min*mEq/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>26 (23-28)</td>
<td>30 (21-32)</td>
<td>27 (24-43)</td>
</tr>
<tr>
<td>Post</td>
<td>27 (21-37)</td>
<td>26 (20-28)*</td>
<td>24 (20-33)</td>
</tr>
<tr>
<td><strong>% NEFA suppression AUC (_{0-180})</strong>, (1000<em>min</em>%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>10.1 (8.8-11.2)</td>
<td>9.5 (7.8-10.6)</td>
<td>8.5 (8.1-10.1)</td>
</tr>
<tr>
<td>Post</td>
<td>8.2 (6.8-8.8)</td>
<td>9.8 (9.2-10.7)*</td>
<td>8.1 (6.8-9.8)</td>
</tr>
<tr>
<td><strong>% NEFA suppression AUC (_{0-90})</strong>, (1000<em>min</em>%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>5.1 (4.4-5.7)</td>
<td>4.5 (3.8-5.7)</td>
<td>4.2 (3.6-5.0)</td>
</tr>
<tr>
<td>Post</td>
<td>4.0 (3.3-4.7)</td>
<td>5.5 (4.0-5.8)*</td>
<td>5.4 (4.2-5.5)*</td>
</tr>
<tr>
<td><strong>Coefficient of glucose disappearance</strong> ((1000*min^{-1}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>12 ± 2</td>
<td>12 ± 1</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Post</td>
<td>11 ± 2</td>
<td>13 ± 1</td>
<td>13 ± 2</td>
</tr>
</tbody>
</table>
Table 4.4  Body weight, RER, heat production, and heat production per metabolic body size (BW$^{0.75}$) for 12 obese cats before and after 5 weeks of treatment with placebo, 1 mg/kg pioglitazone, or 3 mg/kg pioglitazone. Values with non-normal distribution are expressed as median (IQR), with the exception of body weight, which is expressed as median (range); normally distributed values are expressed as mean ± SE. Mean ± SE daily food intake is also shown for each treatment period. * = significant change from pre-treatment value vs. change with placebo ($P<0.05$).

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>1 mg/kg pioglitazone</th>
<th>3 mg/kg pioglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>6.2 (5.0-9.7)</td>
<td>6.2 (5.2-9.7)</td>
<td>6.2 (5.4-9.9)</td>
</tr>
<tr>
<td>Post</td>
<td>6.3 (5.0-9.9)</td>
<td>6.2 (5.0-9.7)</td>
<td>6.3 (5.3-9.9)</td>
</tr>
<tr>
<td>Average daily food intake (g)</td>
<td>62 ± 3</td>
<td>62 ± 4</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>RER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>0.75 (0.74-0.77)</td>
<td>0.76 (0.75-0.76)</td>
<td>0.75 (0.74-0.77)</td>
</tr>
<tr>
<td>Post</td>
<td>0.75 (0.74-0.77)</td>
<td>0.74 (0.74-0.76)*</td>
<td>0.75 (0.74-0.76)*</td>
</tr>
<tr>
<td>Heat production (kcal/h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>11.1 ± 0.4</td>
<td>11.4 ± 0.6</td>
<td>11.1 ± 0.4</td>
</tr>
<tr>
<td>Post</td>
<td>10.9 ± 0.5</td>
<td>11.1 ± 0.5</td>
<td>10.9 ± 0.5</td>
</tr>
<tr>
<td>Heat production per MBS (kcal/h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>2.8 (2.5-3.1)</td>
<td>2.8 (2.5-3.1)</td>
<td>2.7 (2.5-3.0)</td>
</tr>
<tr>
<td>Post</td>
<td>2.7 (2.5-3.0)</td>
<td>2.7 (2.6-3.0)</td>
<td>2.8 (2.4-3.0)</td>
</tr>
</tbody>
</table>

Table 4.5  Mean ± SD $C_{max}$, AUC$_{0\text{-inf}}$, and $t_{1/2}\lambda_d$ estimated from the concentration-time profiles of obese cats dosed with 1 mg/kg or 3 mg/kg oral pioglitazone (Actos™) (both n=11). * = significant difference vs. 1 mg/kg dosage ($P<0.05$).

<table>
<thead>
<tr>
<th></th>
<th>1 mg/kg pioglitazone</th>
<th>3 mg/kg pioglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>997 ± 208</td>
<td>1528 ± 527*</td>
</tr>
<tr>
<td>AUC$_{0\text{-inf}}$ (h*μg/mL)</td>
<td>7.44 ± 2.25</td>
<td>11.9 ± 5.4*</td>
</tr>
<tr>
<td>$t_{1/2}\lambda_d$ (h)</td>
<td>3.65 ± 0.65</td>
<td>3.67 ± 0.56</td>
</tr>
</tbody>
</table>
4.7 References


Chapter V

Investigation of $^1$H MRS for quantification of hepatic triglyceride in lean and obese cats

5.1 Abstract

$^1$H magnetic resonance spectroscopy ($^1$H MRS) is the preferred technique for noninvasive quantification of hepatic triglyceride in humans. Domestic cats are subject to liver lipid accumulation, but MRS has not been investigated for quantification of liver fat in cats. The purpose of this project was to investigate a technique for $^1$H MRS measurement of hepatic triglyceride in lean and obese cats. Hepatic $^1$H MRS was performed, using a 3T imaging unit and a single-voxel spin-echo spectroscopy sequence with respiratory synchronization, on 6 lean (3.3-4.6 kg) and 12 obese cats (5.2-9.8 kg). In addition, liver samples from 4 lean (2.3-4.6 kg) and 5 obese cats (5.3-7.6 kg) were used for chemical determination of triglyceride content. Median liver fat percentages in lean and obese cats were 1.3% and 6.8%, respectively, by $^1$H MRS. In cats with samples analyzed by chemical assay, median liver fat percentages were 1.7% (lean) and 6% (obese). Chemical assay results support the biological plausibility of measurements obtained using $^1$H MRS. Full validation of the $^1$H MRS method may be necessary before other conclusions can be drawn; however, this report should provide a foundation for the further development of spectroscopic techniques for studying hepatic lipid accumulation in cats.

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2 Submitted in modified form, as a short communication, to Research in Veterinary Science.
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*Biomedical Imaging Center, Beckman Institute for Advanced Science and Technology, University of Illinois, Urbana, IL, 61801, USA
bDepartment of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois, Urbana, IL, 61802, USA).
5.2 Introduction

Magnetic resonance spectroscopy (MRS) is a sensitive, noninvasive technique that can be used to investigate the molecular composition of living tissues. Like magnetic resonance imaging (MRI), MRS is performed by detecting electrical signals produced by isotopic nuclei in a large external magnetic field, after they are excited by a radio frequency pulse (Castillo et al., 1996). MRS exploits the fact that in the presence of the magnetic field, atoms exhibit shifts in their Larmor, or resonant, frequencies that depend on their location within a molecule. The electrical signals they generate are probed over a range of frequencies, producing a spectrum that gives quantitative information about the chemical content of the sample. Various isotopes can be used for MRS; among them, the hydrogen nucleus (\(^{1}\)H) is abundant in biological systems and is particularly sensitive to radiofrequency excitation (Castillo et al., 1996). \(^{1}\)H MRS, or proton spectroscopy, can distinguish the protons of water molecules from those of fatty acids in a given volume of tissue. Thus, it provides an accurate determination of tissue fat content (Longo et al., 1995; Cassidy et al., 2009).

Within the past 20 years, \(^{1}\)H MRS has become widely used in human clinical research for noninvasive measurement of hepatocellular triglyceride (Fabbriini et al., 2009; Schwenzer et al., 2009). Lipid accumulation in hepatocytes is a feature of human obesity and type 2 diabetes (Wanless and Lentz, 1990; Kelley et al., 2003). It is also the defining characteristic of human non-alcoholic fatty liver disease (NAFLD), a chronic hepatopathy with variable progression from simple steatosis to severe steatohepatitis and fibrosis (Lebovics and Rubin, 2011). For
identification of steatosis, magnetic resonance techniques, including $^1$H MRS, are more sensitive and specific than ultrasound (US) or computed tomography (CT) (Lee et al., 2008). As well, $^1$H MRS-derived measurements of liver fat correspond closely with those obtained by chemical triglyceride assay of liver biopsies (Thomsen et al., 1994; Szczepaniak et al., 1999). Consequently, $^1$H MRS has been used in lieu of biopsy in large patient cohorts to explore associations between high hepatic lipid content and other physiologic derangements such as insulin resistance (Holt et al., 2006; Korenblat et al., 2008). It has also proven particularly useful in longitudinal studies of patients with hepatic steatosis, as it permits serial measurements and evaluation of the results of therapeutic interventions without the risks of biopsy (Bajaj et al., 2003).

Liver triglyceride accumulation occurs in domestic cats as a result of diabetes (Johnson et al., 1986) or prolonged fasting (feline hepatic lipidosis) (Armstrong et al., 2009). Obese cats may also have elevated liver fat, although previous studies on this subject have yielded conflicting results (Dimski et al., 1992; Biourge et al. 1994a; Nicoll et al. 1998b; Blanchard et al., 2001). The ability to noninvasively quantify liver fat in cats would facilitate study of pathophysiologic mechanisms and novel therapeutics for excess hepatic lipid storage in this species. Although ultrasound or computed tomography may be used as screening tools for severe hepatic lipid accumulation (Nicoll et al., 1998a; Nakamura et al., 2005), neither method is ideal for repeated quantitative assessment. $^1$H MRS has been used to demonstrate elevated triglyceride in the muscle of obese cats (Wilkins et al., 2004). However, it has not previously been investigated for quantification of feline liver fat. The purpose of this project was to explore a technique for $^1$H MRS measurement of hepatic triglyceride in cats.
5.3 Materials and Methods

Animals

Eighteen adult purpose-bred Domestic Shorthair cats were used for $^1$H MRS. Cats were classified as lean (n=6) or obese (n=12) based on percent gain of adult lean body weight. Obese cats had gained >50% of their adult lean body weight (median 98%; range 54%-109%), and had been obese for > 1 year. Median weight of the obese cats was 6.2 kg (range 5.2-9.8 kg); median weight of the lean cats was 3.5 kg (range 3.3-4.6 kg). Of the obese cats, 6 were neutered males and 6 were spayed females, and median age was 5 years (range 5-7 years). Of the lean cats, 4 were neutered males and 2 were spayed females, and all were 4-5 years of age. Cats were housed in the University of Illinois animal care facility and were fed a commercial dry feline maintenance diet (Pro Plan Chicken & Rice Formula, Nestlé Purina) once daily. All procedures involving the cats were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee.

Tissues

Liver samples from 4 lean and 5 obese healthy cats, euthanized for reasons unrelated to the current project, were used for chemical triglyceride assay. Samples had been snap-frozen in liquid nitrogen immediately after collection and stored at -80 C. Obese cats from which the samples were obtained (1 neutered male, 4 spayed females; 5 to 12 years of age) had a median weight of 6.7 kg (range, 5.3-7.6 kg). Lean cats (3 neutered males, 1 spayed female; 4 to 8 years of age) had a median weight of 3.9 kg (range, 2.3-4.6 kg).
For spectroscopic examination, cats were sedated with tiletamine-zolazepam (Telazol, Pfizer Animal Health). Scanning was performed in a 3T scanner (Magnetom Trio, Siemens Medical Systems). The cats were placed in a supine position in a custom-made holder with a semi-circular cross section. An infant respiratory belt (Lafayette Instrument Company) was positioned over the abdomen of the cats to monitor breathing. Imaging was performed with a flexible body matrix coil and a spine matrix coil which were positioned above and below the cats, respectively. Ear plugs were placed in the ears as protection from scanner noise.

The cats were first scanned using T<sub>1</sub>-weighted scans obtained with a 2D flash gradient echo sequence, with 9-12 slices in the sagittal, dorsal, and transverse orientations. Slice thickness was 7 mm, and slices covered a field of view of 200 mm x 200 mm with a resolution of 256 points (TR = 6.8 ms, TE = 4 ms, flip angle = 20°). For most cats, additional T<sub>2</sub>-weighted scans were performed using a 2D turbo spin echo sequence, with respiratory gating based on signal from the respiratory belt, and 2 averages. Scans were performed in three orientations: dorsal (17 slices, slice thickness: 3 mm, 180 mm x 108 mm, 256 x 154 voxels, flip angle = 120°, TR = 3600 ms, TE = 103 ms), sagittal (15 slices, slice thickness: 3 mm, 180 mm x 107 mm, 192 x 114 voxels, flip angle = 120°-140°, TR = 3600 ms, TE = 107 ms), and transverse (16 slices, slice thickness: 3 mm, 220 mm x 165 mm, 192 x 144 voxels, flip angle = 120°, TR = 3600 ms, TE = 103 ms).

Scanning parameters were adjusted for some cats so that the Specific Adsorption Rate (SAR) estimation provided by the scanner remained within First Level Controlled Operating Mode. For some cases, to meet this requirement, the number of slices was reduced and the slice
thickness was increased to 8 mm, or the resolution was decreased, to avoid significant heating due to radiofrequency deposition.

The relative concentrations of fat and water were assessed by performing single voxel magnetic resonance spectroscopy imaging (MRSI) measurements, TR = 3000 ms, TE = 50 ms, 20 averages, 8x8x8 mm, flip angle 90°, 1024 points, bandwidth 2000 Hz, with respiratory gating based on signal from the respiratory belt. A combination of the T1 and the T2-weighted scans was used to select locations for spectroscopy. Spectroscopy was performed at two different locations within the liver, and voxels were positioned as far as possible from the gallbladder, surrounding fat, lung, and vascular structures. The latter approach minimized distortions due to chemical shift artifact. This artifact stems from the fact that the excitation pulse causes the volume of excited fat to be displaced relative to the volume of the excited water. By default, the selected volume provided by the scanner control software corresponds to the location of the excited water pulse. However, this setting can be modified by changing the frequency of the excitation with the Delta Frequency parameter provided in the sequence protocol. When this parameter is set to the difference between the fat and water resonances, approximately -3.4 to -3.7 ppm, the selected region will show the location of the excited fat. For an ideal measurement, the voxel would be placed in a region of homogeneous liver that is sufficiently far from fat, lung, and vascular structures that the measurement would be independent of the Delta Frequency parameter. This assumption was tested at each of the two locations by performing three measurements per location, in which the Delta Frequency parameter was set at 0, -1.9 ppm, and -3.8 ppm.

The relative proton densities of fat and water, $\rho_F$ and $\rho_W$, were calculated from the spectra using commercially available software (LCModel; Provencher, 2001). The proton
density of fat was taken from the main fat peak at 1 ppm that includes sub-peaks expected at 0.9, 1.3, and 1.6 ppm. Other available fat peaks were typically much smaller and were neglected in our analysis. The fat fraction, FTSA, of the proton density was calculated from

$$\text{FTSA} = \frac{\rho_F}{\rho_F + \rho_W},$$

where $\rho_F$ and $\rho_W$ are the proton densities of fat and water, respectively.

When the range of the FTSA values calculated from a single location was greater than 0.03, measurements from that voxel were not considered to be independent of changes in the Delta Frequency parameter, and all measurements from the voxel were excluded from analysis. Mean values of FTSA for each cat were obtained by averaging all non-excluded measurements.

The average values of FTSA calculated from the spectroscopy measurements were converted to a volume or weight fraction of triglyceride in the liver ($\varphi_f$; expressed as percent) according to the following equation:

$$\varphi_f = \frac{100 \times \text{FTSA}}{1.138 - 0.508 \times \text{FTSA}}.$$

This equation is derived from those validated for $^1$H MRS in humans by Longo et al. (1995).

The original series of equations incorporated the ratio of spectroscopically detectable lipid protons in the 0.5-3.0 ppm range to total lipid protons (0.85), proton densities of water and fat (110 and 111 mol/L, respectively), density of liver tissue (approximately 1 g/L), density of fat in the liver (0.9 g/L), and percent water weight of human liver (0.71 g water/g normal tissue). Because average percent water weight of the liver in cats has been reported as 0.66 g water/g normal tissue (Fenn and Haege, 1940), the latter value was used for calculations in the current investigation. Other factors listed were assumed to be relatively constant among humans and domestic animals.

Total scan time per cat was 20-30 min. Girth (abdominal circumference) was measured immediately caudal to the last rib in sedated cats on the day of scanning. In 4 obese cats, scans
were repeated after 6 weeks of administration of a lactose placebo, as part of a separate study; these data were used to characterize the repeatability of the measurements.

**Chemical triglyceride assay**

Lipids were extracted from liver samples using a modification of the method of Folch (Folch et al., 1957; Kishida et al., 2002). Briefly, 300-900 mg liver samples were homogenized in chloroform:methanol (2:1, v/v) with a rotor-stator homogenizer (Polytron PCU-2-110; Brinkmann Instruments, Inc.). Homogenates were centrifuged at 20 °C at ~ 500 x g for 5-10 min, and the supernatant was decanted, filtered through filter paper (Whatman Filter Paper No. 1, GE Healthcare), and brought to a volume of 25 mL with 2:1 chloroform:methanol. Duplicate aliquots of the extract were evaporated to dryness under nitrogen, and resuspended in isopropanol/Triton-X (90:10). Triglyceride concentration was determined using a colorimetric method (Triglyceride Reagent Kit, Pointe Scientific).

**Statistical analysis**

For both $^1$H MRS and chemical assay data, median liver fat percentage was compared between lean and obese cats using a Mann-Whitney U-test. Mann-Whitney U-tests were one-sided, with $H_a = \eta_{\text{lean}} < \eta_{\text{obese}}$, where $\eta_{\text{lean}}$ and $\eta_{\text{obese}}$ represent median percent liver triglyceride in lean and obese cats, respectively. P-values <0.05 were considered significant.

**5.4 Results**

$^1$H magnetic resonance spectra were successfully obtained in all 18 cats. A representative spectrum, with its corresponding localizing scans and voxel placement, is shown in Figure 5.1.
In three obese cats and one lean cat, fat fraction values from one voxel varied with changes in the Delta Frequency parameter, such that the difference between the highest and lowest measurements was >0.03. This was considered evidence of artifact arising either from motion or from placement of the voxel near areas where fat was not homogeneous. Data from these voxels were excluded from analysis, and average liver fat percentage in these cats was calculated from the three measurements in the remaining voxel.

In the other 14 cats, placement of both voxels was adequate to avoid artifact from motion or chemical shift, and all measurements were used for calculation of mean fat fraction. Maximum difference in liver fat content between the two locations in these cats was 1.2% (lean cats) or 3% (obese cats). Median liver fat percentages in lean and obese cats by spectroscopy were 1.3% (range, 0.6 to 5.7%) and 6.8% (range, 3.9 to 16.4%), respectively. For samples analyzed by chemical assay, median liver fat percentage was 1.7% (range, 0.7 to 2.3%) in lean cats and 6% (range, 2.7 to 7.3%) in obese cats. For both $^1$H MRS and chemical assay measurements, values for liver triglyceride were significantly greater in obese than in lean cats ($p \leq 0.03$). $^1$H MRS-determined liver fat percentages in relation to body weight and girth are shown in Figures 5.2A and 5.2B, respectively. Values for liver triglyceride vs. body weight in the separate group of cats with samples analyzed by chemical assay are shown in Figure 5.3.

In the 4 obese cats in which scans were repeated after 6 weeks, mean coefficient of variation for the two determinations of average liver fat percentage was 7.4%, and maximum coefficient of variation was 12.3%. A mean coefficient of variation of 8.4% has been reported in humans for two temporally separated determinations on the same day (Szczepaniak et al., 2005).
5.5 Discussion

A previous publication involving chemical liver triglyceride measurement in wedge biopsies from cats fed a maintenance diet reported a mean liver triglyceride content of 1% for 5 cats of ideal body weight (Hall et al., 1997). A separate publication, using similar methods, reported liver fat percentages of 1.5%-6% for 4 groups of 3 obese cats (≥ 40% overweight compared to lean cats of the same length and body type) (Biourge et al., 1994b). Other quantitative analyses of feline liver fat (Blanchard et al., 2002; Nicoll et al., 1998a) have produced varying results, perhaps because of differences in techniques used to measure triglyceride, methods of biopsy procurement, definitions of obesity, and diets fed to obese cats prior to assessment. To gain further information regarding the expected range for liver triglyceride in obese compared to lean cats, the present investigation utilized chemical assay to measure liver triglyceride percentage in a small group of both lean and obese cats fed a maintenance diet. Chemical triglyceride assay is considered the most accurate method of determining lipid content in liver biopsies, as it is capable of measuring the lipid in vacuoles that are too small to be recognized on histological evaluation (Longo et al., 1995; Szczepaniak et al., 2005). The results of chemical assay in liver samples from this study support the premise that hepatic triglyceride accumulation occurs in feline obesity, and that liver fat content in obese cats is frequently >5%. In humans, >5% liver fat is considered abnormal, and is adequate for a classification of hepatic steatosis (Szczepaniak et al., 2005).

In dogs, rabbits, and humans, $^1$H MRS has been validated against chemical triglyceride assay by comparing the results of the two assessments in the same individual (Longo et al., 1995; Szczepaniak et al., 1999). The latter procedure was not replicated in the cats of this study, as it was not possible to obtain liver biopsies from the same cats that were scanned. Nonetheless, the
\(^1\)H MRS method described here appears repeatable, based on consistency of measurements in the same cats several weeks apart. As well, measurements obtained by \(^1\)H MRS differentiated well, in general, between cats classified as lean and those classified as obese. An interesting finding was a \(^1\)H MRS measurement of 5.7% liver fat in one of the six lean cats. This lean cat also had a surprisingly high body fat percentage (27%) on DEXA scanning, despite a body weight and girth that were comparable to those of the other lean cats. In humans, although there is a general positive relationship between obesity and high liver fat, 3-15% of humans with normal body weight have elevated liver triglyceride (Fabbrini et al., 2010). Genetic background, habitual activity level, and diet are all known to alter the relationship between liver fat percentage and adiposity (Browning et al., 2004; Westerbacka et al., 2005; Perseghin et al., 2007; Romeo et al., 2008). A “metabolically obese normal-weight” phenotype has been described in people who have normal BMI but high body fat and ectopic lipid deposition (Karelis et al., 2004); it is conceivable that a similar condition occurs in cats.

In summary, measurements produced by the \(^1\)H MRS technique described here are biologically plausible, based on the results of chemical assay in a separate group of lean and obese cats. Full validation of the method may be necessary before other conclusions can be drawn. However, this report contributes to existing knowledge regarding expected liver fat content in feline obesity, and should provide a foundation for further development of quantitative spectroscopic techniques to study hepatic lipid accumulation in cats.

**Acknowledgments**

We are grateful for the valuable technical assistance of Dr. Mohamedain Mahfouz, Holly Tracy, Nancy Dodge, and Dr. Tracey Wszalek. Funding for this study was provided by the Biomedical Imaging Center, Beckman Institute, University of Illinois, and by the Morris Animal Foundation.
5.6 Figures and Legends

Figure 5.1 Representative spectrum (D), with corresponding localizing scans and voxel placement (A: sagittal view; B: dorsal view; C: transverse view) from hepatic $^1$H MRS in an obese cat (body weight 6.5 kg; $^1$H MRS-measured liver triglyceride 6.5%). Peaks at 1.0-2.0 ppm and 4.0-5.0 ppm correspond to lipid and water protons, respectively. Objects in the transverse view are phantoms used for a separate experiment.
Figure 5.2 $^1$H MRS-determined liver fat percentage relative to body weight (A) and girth (B) in 6 lean and 12 obese cats.

Figure 5.3 Chemically determined liver fat percentage relative to body weight in 4 lean and 5 obese cats.
5.7 References


Chapter VI

Conclusion

6.1 Conclusion

The goals of the work described in this dissertation were 1) to determine the metabolic effects of pioglitazone in cats, in order to assess its potential for treatment of feline DM, and 2) to investigate a technique for noninvasive measurement of feline liver fat. As reviewed in Chapter 2, DM is a relatively common endocrinopathy in cats, with treatment options limited to insulin and one oral insulin secretogogue. Considering the similarities between type 2 DM in humans and DM in cats, it seemed reasonable to continue exploration of the TZDs, which improve both insulin sensitivity and β-cell function in human type 2 DM, in cats. Feline hepatic lipidosis has a poorly understood pathogenesis and no pharmacological treatment. The noninvasive technology of $^1$H MRS, which is used in human clinical research for determination of liver fat content, offered a possible alternative to biopsy for eventual study of the effects of therapeutic interventions on this disease.

Chapter 3 of this dissertation describes a single-dose pharmacokinetic study of pioglitazone that was carried out in lean and obese cats. Results indicated that pioglitazone was rapidly absorbed, with an average bioavailability of 55%, and that plasma pioglitazone concentrations considered therapeutic in humans were achievable in cats with oral administration. There were no differences in pharmacokinetic parameters for pioglitazone
between lean and obese cats. The data obtained from this study provided a basis for dosage selection in further investigations.

In the work discussed in Chapter 4, 1 and 3 mg/kg dosages of pioglitazone were administered orally to 12 obese, insulin-resistant cats, in a placebo-controlled, 3-way crossover design. Six weeks of 3 mg/kg pioglitazone lowered insulin concentrations in the obese cats during glucose tolerance testing, and significantly increased insulin sensitivity with respect to both glucose clearance and NEFA disposal. Pioglitazone also appeared to have a favorable effect on lipid metabolism in obese cats, as it increased NEFA suppression during the initial portion of IVGTT, and lowered cholesterol and triglyceride concentrations. Based on clinical and laboratory evaluation, no overt toxicity of the drug was evident in otherwise healthy obese cats. This is in contrast to results for metformin, a hepatic insulin sensitizer which is relatively safe in humans but produced unacceptable adverse effects in both healthy and diabetic cats (Nelson et al., 2004).

In the study described in Chapter 5, liver fat in 12 obese and 6 lean cats was measured by $^1$H MRS, and average liver fat percentages of 6.8% and 1.3% were obtained for obese and lean cats. These results were supported by chemical determination of liver fat percentage in a separate group of cats. The $^1$H MRS technique employed differentiated well between lean and obese cats in terms of liver fat, and appeared repeatable in 4 cats scanned 6 weeks apart. In addition to supplementing existing knowledge regarding liver fat content in feline obesity, this study provided a foundation for further development of quantitative spectroscopic techniques to study hepatic lipid accumulation in cats.

Overall, the results of these investigations demonstrate a positive effect of 3 mg/kg pioglitazone on insulin sensitivity and lipid metabolism in obese cats. Further study of the drug
might entail clinical trials in cats with DM or other lipid metabolic disorders. In diabetic cats, an interesting possibility is that pioglitazone might improve β-cell function, as it does in human diabetes, perhaps leading to improved long-term glycemic control and/or an increased chance of diabetic remission. Studies in diabetic cats could incorporate the recently developed feline proinsulin assay (Kley et al., 2008), in conjunction with insulin concentrations and clinical status, to evaluate β-cell functional improvement. The proinsulin:insulin ratio has been used as an index of β-cell dysfunction in human diabetics, and decreases with pioglitazone or other TZD treatment (Ovalle & Bell, 2004; Wallace et al., 2004).

With respect to the technique for $^1$H MRS in cats, although it could not be used to assess the effects of pioglitazone in the work described here, it may be helpful in future investigations, either of dietary components or of pharmacologic agents for reducing liver lipid accumulation. Limitations of $^1$H MRS include cost, the need for deep sedation or anesthesia of subjects, and the requirement for spectroscopic software and expertise. However, when appropriate software and an individual with knowledge of spectroscopy are available, $^1$H MRS can be carried out on any commercially available scanner with a field strength of 1.5 T or higher (Schwenzer et al., 2009). If the method can be appropriately validated, it may ultimately prove useful for exploring the pathophysiology associated with feline hepatic lipid accumulation, as well as for examining novel therapies for feline hepatic lipidosis.
6.2 References


LIST OF ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>$\lambda_z$</td>
<td>terminal elimination rate constant</td>
</tr>
<tr>
<td>$^1$H MRS</td>
<td>proton magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>2D</td>
<td>2-dimensional</td>
</tr>
<tr>
<td>A</td>
<td>y-intercept for the distribution phase</td>
</tr>
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<td>ACS</td>
<td>acyl coA synthase</td>
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<td>AF2</td>
<td>activation function 2</td>
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<td>AIC</td>
<td>Akaike's information criterion</td>
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<td>ALP</td>
<td>alkaline phosphatase</td>
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<td>AMP</td>
<td>adenosine monophosphate</td>
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<tr>
<td>aP2/FABP4</td>
<td>adipocyte Protein 2/fatty acid binding protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<td>AUC$_{0\text{-inf}}$</td>
<td>area under the curve from time 0 to infinity</td>
</tr>
<tr>
<td>AUMC$_{0\text{-inf}}$</td>
<td>area under the moment curve from time 0 to infinity</td>
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<tr>
<td>B</td>
<td>y-intercept for the elimination phase</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>C/EBP$\alpha$</td>
<td>CCAAT enhancer binding protein alpha</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CD36</td>
<td>cluster of differentiation 6</td>
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<td>Cdk5</td>
<td>cyclin-dependent kinase 5</td>
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<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
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<tr>
<td>CI/F</td>
<td>oral clearance</td>
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<tr>
<td>CI$_s$</td>
<td>systemic clearance</td>
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<tr>
<td>$C_{\text{max}}$</td>
<td>maximum plasma concentration</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>cytochrome P450 2C8</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>cytochrome P450 3A4</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DEXA</td>
<td>dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>DKA</td>
<td>diabetic ketoacidosis</td>
</tr>
<tr>
<td>DM</td>
<td>diabetes mellitus</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EHC</td>
<td>euglycemic-hyperinsulinemic clamp</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>F</td>
<td>bioavailability</td>
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<tr>
<td>FATP1</td>
<td>fatty acid transport protein 1</td>
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<tr>
<td>FOXO1</td>
<td>forkhead box protein O1</td>
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<tr>
<td>FS</td>
<td>fractional shortening</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FTSA</td>
<td>fat-to-total-signal peak area</td>
</tr>
<tr>
<td>GLUT2</td>
<td>glucose transporter 2</td>
</tr>
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<td>GLUT4</td>
<td>glucose transporter 4</td>
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<tr>
<td>HCl</td>
<td>hydrochloride</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
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<td>HL</td>
<td>hepatic lipodosis</td>
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<tr>
<td>HMBS</td>
<td>heat per metabolic body size</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>IGT</td>
<td>impaired glucose tolerance</td>
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<td>IL-6</td>
<td>interleukin-6</td>
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<td>IQR</td>
<td>interquartile range</td>
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<td>IR</td>
<td>insulin receptor</td>
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<td>insulin receptor substrate</td>
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<td>IS</td>
<td>internal standard</td>
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<td>IV</td>
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<td>IVSs</td>
<td>interventricular septal thickness (systole)</td>
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<td>JNK</td>
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<td>potassium</td>
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<tr>
<td>LA/Ao</td>
<td>left atrial/aortic ratio</td>
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<tr>
<td>LC-FA CoA</td>
<td>long chain fatty acyl coenzyme A</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>limit of detection</td>
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<td>LVPWd</td>
<td>left ventricular posterior wall (diastole)</td>
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<td>MafA</td>
<td>musculoaponeurotic fibrosarcoma oncogene homolog A</td>
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<td>MAP kinase</td>
<td>mitogen-activated protein kinase</td>
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<td>MRT</td>
<td>mean residence time</td>
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<td>NI_i</td>
<td>non-insulin-dependent NEFA concentration at basal</td>
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<td>NLME</td>
<td>nonlinear mixed-effects</td>
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<td>( P_2 )</td>
<td>rate constant of time lag between supra-basal changes in insulin concentrations and insulin action, ( X(t) )</td>
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$P_{2N}$  

- time-lag between supra basal changes in plasma insulin and NEFA suppression

PDX-1  

- pancreatic and duodenal homeobox 1

PEPCK  

- phosphoenolpyruvate carboxykinase

PI(3)K  

- phosphatidylinositol (3) kinase

PKC  

- protein kinase C

PPARγ  

- peroxisome proliferator activated receptor gamma

PPRE  

- PPAR response element

PRESS  

- point-resolved echo spin sequence

PZI  

- protamine zinc insulin

Rₐ  

- rate of appearance

RER  

- respiratory exchange ratio

RXR  

- retinoid X receptor

SAR  

- Specific Adsorption Rate

SD  

- standard deviation

SEM  

- standard error of the mean

$S_G$  

- fractional glucose clearance at basal insulin

$S_I$  

- insulin sensitivity of fractional glucose disappearance

$S_{IN}$  

- sensitivity of fractional NEFA inhibition to changes in plasma insulin around basal

T  

- tesla

t₁/₂α  

- distribution half-life

t₁/₂β  

- terminal elimination half-life

t₁/₂k₁₀  

- elimination half-life

t₁/₂λ₂  

- terminal elimination half-life

$T_1$  

- spin-lattice relaxation time constant

$T_2$  

- spin-spin relaxation time constant

$T_{max}$  

- time to maximum plasma concentration

TE  

- echo time

TR  

- repetition time

TNF-α  

- tumor necrosis factor alpha

TZD  

- thiazolidinedione

UCP1  

- uncoupling protein 1

$V_6$  

- volume of distribution of the terminal elimination phase

$V_c$  

- volume of distribution of the central compartment

$V_d/F$  

- apparent oral volume of distribution

$V_G$  

- apparent glucose distribution volume

VLDL  

- very low-density lipoprotein

$V_{ss}$  

- volume of distribution at steady state

$V_p/F$  

- apparent oral volume of distribution