EVEN A LITTLE IS TOO MUCH: A LONGITUDINAL STUDY OF WEIGHT GAIN IN THE CAT

BY

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THESIS

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Abstract

Obesity and insulin resistance has increased in the last three decades among humans and cats and has been associated with an increase in the inflammatory response and oxidative stress in people. The purpose of this study was to determine if an increase in fat mass was associated with an increased inflammatory response and oxidative stress, leading to insulin resistance and abnormal insulin secretion. Twenty cats of equal gender distribution were used for the study; 10 were fed a control diet (C) and 10 were fed the same diet containing an antioxidant mixture (AOX). The following parameters were measured at 0%, 10%. 30% and 60% weight increase: body mass index (BMI), girth, total fat, food intake, glucose and insulin concentrations before and after an intravenous glucose tolerance test, baseline cholesterol, HDL, triglyceride, non-esterified fatty acids (NEFAs), catalase, glutathione peroxidase, superoxide dismutase, interleukin-6 (IL-6), Interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-alpha), respiratory exchange ration (RER), and heat production.

Body mass index (BMI), girth, total fat, and energy intake increased significantly with increasing body weight. There was no difference in glucose baseline concentrations as a result of body weight increase. However, at 60% body weight increase the AUC for glucose increased significantly in cats fed AOX, indicating glucose intolerance. A significant decrease was seen in insulin sensitivity with increasing body weight. Baseline insulin concentrations and total AUC increased significantly with increasing body weight, BMI and girth indicating insulin resistance even at a 10% body weight increase. Triglycerides and NEFAs increased significantly with increasing body weight, indicating a possible increase in VLDL and a decrease in lipoprotein lipase. The enzyme activity of catalase, glutathione peroxidase, and superoxide dismutase did not change significantly with increasing body weight, indicating a lack of oxidative stress. The

cytokines IL-6, IL-1, and TNF-alpha did not change significantly with increasing body weight, indicating no inflammatory response with increasing body weight. The RER increased significantly at 30 and 60% body weight increase, indicating a decrease in fat oxidation. A significant increase in heat production with increasing body weight was seen, indicating increased energy production, likely due to the increase in food intake.

In conclusion, an increase in obesity does not elicit an inflammatory response in cats, despite the fact that it leads to insulin resistance even at a 10% body weight increase. These results may in part explain the fact that obese cats do no exhibit atherosclerosis, hypertension or other signs of the metabolic syndrome observed in obese human subjects.

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Chapter 1 Introduction

Obesity is a risk factor for diabetes in humans and cats and approximately 35-45% of cats are obese (Lund et al 2005). Research has shown that obese cats have two defects: they are insulin resistant (Hoenig et al 2006 and 2007), and they show an abnormal insulin secretion pattern (Hoenig et al 2002). Chronic oxidative stress is associated with obesity and insulin resistance in human subjects (Evans et al 2003). However, it is not known whether oxidative stress causes obesity or whether obesity and insulin resistance cause oxidative stress. Oxidative stress occurs when there is an imbalance between free radicals and antioxidants. Lipid peroxidation is a type of oxidative stress where lipids are oxidized into lipid radicals and is characterized by changes in enzyme markers such as catalase, superoxide dismutase, and glutathione peroxidase, among others. During the postprandial phase, lipoproteins are prone to lipid peroxidation because they are enriched with triglycerides, and these oxidized lipoprotein particles are phagocytosed by macrophages via the scavenger receptor however, macrophages cannot metabolize the lipoproteins and therefore they accumulate inside the cytoplasm, resulting in atherosclerosis in people (Berliner et al 1995). The oxidized lipoproteins can also be chemotactic for monocytes and stimulate the expression of cytokines. The stimulation of the inflammatory response has been shown to induce insulin resistance. We hypothesized that the increase in fat mass with obesity leads to an increase in oxidative stress in adipose tissue (Figure 1. step 1). During postprandial lipemia, the delivery of dietary fats to adipose tissue is accompanied by the exposure of intestinal lipoproteins to increased oxidative stress, resulting in oxidative modification of plasma lipoproteins (Figure 1. step 2). In the absence of effective antioxidant protection, excess oxidized modified lipoproteins may result in macrophage recruitment both in adipose tissue and peripheral tissues (Figure 1. step 3). The accumulation of macrophages will,

in turn lead to the secretion of inflammatory cytokines and adipokines (Figure 1. step 4-7). This proposed sequence would initiate the vicious cycle leading to insulin resistance, abnormal insulin secretion (Figure 1. step 4 and 6) and ultimately to diabetes.

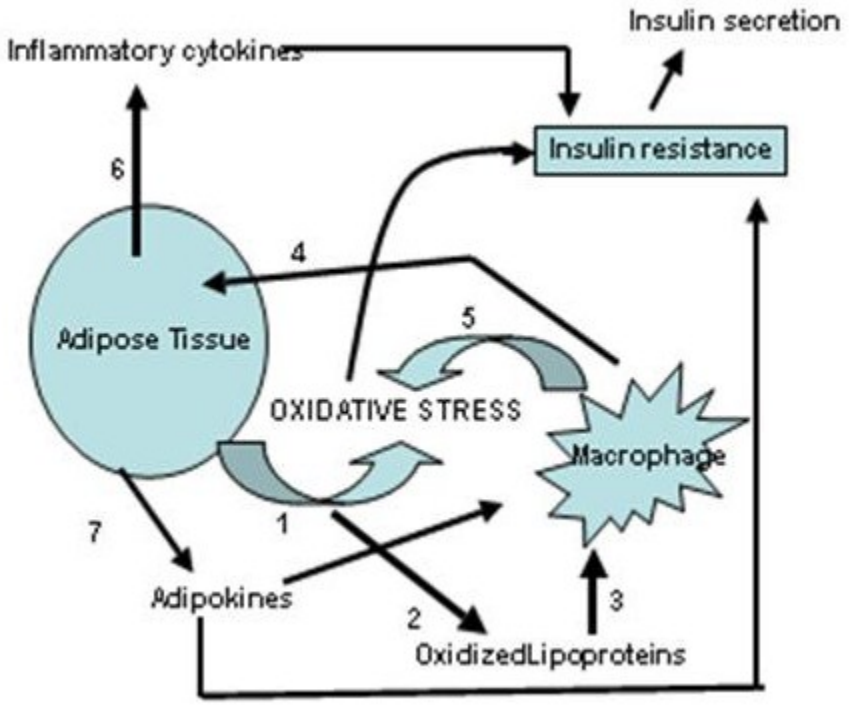

Figure 1: Temporal development of insulin resistance**.**

The goals of this study were:

1. To demonstrate that an increase in fat mass is associated with a shift in oxidative stress and to characterize the effect of increased fat mass on the modulation of the antioxidant protection enzyme as assessed by changes in the activity of antioxidant enzyme catalase, glutathione peroxidase, and superoxide dismutase.

2. To correlate the gradual changes in insulin resistance and in factors leading to insulin resistance: free fatty acids, and inflammatory cytokines (IL-6, IL-1, TNF-alpha), were measured with the increase in fat mass.

3. To demonstrate that the increase in fat mass is associated with a change in energy expenditure.

4. To demonstrate that supplementation of the diet with antioxidants can reduce oxidative stress.

Chapter 2 Literature Review

Lipids

Lipids are composed of mainly carbon and hydrogen molecules with some oxygen containing functional groups. They are hydrophobic and therefore insoluble in water. Lipids circulate through the blood via molecules called lipoproteins that consist of phospholipids, cholesterol and proteins called apoproteins (Gibbons 2004). Plasma lipoproteins are synthesized in the intestine and in the liver (Field 1995). There are five main types of lipoproteins involved in lipid metabolism and they differ in the amount of triglycerides and cholesterol as well as different kinds of proteins. The different kinds of lipoproteins are referred to as chylomicrons, very low density lipoproteins (VLDLs), intermediate density lipoproteins (IDLs), low density lipoproteins (LDLs), and high density lipoproteins (HDLs). These lipoproteins are further divided into subclasses and differentiated by their particle size. For example, cats and humans have two different kinds of HDLs, HDL_2 and HDL_3 however, cats have higher concentration levels of HDL than humans (Demacker 1988). LDL particle sizes vary among humans (Scheffer et al 1997) and two main particle sizes have been identified, large buoyant LDL particles and small dense LDL particles (Austin and Krauss 1986). Cats have also shown differences in LDL particles depending on their body mass index (Jordan et al 2008). In cats as in people, VLDLs are the largest lipoprotein, followed by LDL and HDL (Jordan et al 2008). Even though cats have higher levels of HDL than humans do, they are still a good model for human comparison because their LDL composition is very similar and they both have the same sub-fractions of HDL (Demacker 1987).

The most biologically important groups of lipids in animals are fats, phospholipids, and steroids. These lipids can be used for energy storage, structural components of cellular

membranes, and cell signaling. Triglycerides are the most abundant lipids in animals and can come from diet, synthesis in the liver, and storage supplies in adipose tissue (Solomon et al 2002). They contain twice the amount of energy as carbohydrates and protein, i.e. 1 gram of fat yields 9 kcal (37kJ), 1 gram of carbohydrate yields 3.75 kcal (16kJ), and 1 gram of protein yields 4k cal (17kJ) (Gibbons 2004). Therefore, they are important storage units of energy.

Exogenous Lipid Metabolism

Exogenous or dietary triglycerides pass through the stomach and are digested in the duodenum by pancreatic lipase into monoglycerides, diglycerides, glycerol and fatty acids (Solomon et al 2002). Bile helps in the digestive process by acting as an emulsifier and breaking down large masses of fat into smaller droplets, this allows the pancreatic lipase to attack it more easily. After triglycerides are broken down into smaller components, they combine with bile salts to form micelles and are then absorbed by epithelial cells lining the intestine. In the epithelial cells, fatty acids and glycerol are re-esterified into triglycerides and packaged into lipoproteins. The lipoprotein formed from the components of the micelle in the epithelial cells is called a nascent chylomicron and is responsible for transporting exogenous triglycerides to other cells. (Cooper 1997). Nascent chylomicrons are mainly composed of triglycerides but contain some cholesterol, along with phospholipids and apoprotein B48 (Cooper 1997). The nascent chylomicrons circulate through the lymphatic system and into the blood where they become 'mature" by receiving apoprotein C and apoprotein E from high density lipoproteins (HDLs) (Bauer 2004). The chylomicrons circulate through the blood entering capillary walls of adipose and muscle tissue (Bauer 2004). The apoprotein C present on the chylomicron activates an enzyme called lipoprotein lipase that is attached to the endothelial lining of capillaries via a heparin sulphate-proteoglycan (HSPG) (Braun 1992 and Mead et al 2002). Lipoprotein lipase is

made in parenchymal cells and is then transported to the endothelial lining of capillaries. Activated lipoprotein lipase will break down the triglycerides in the chylomicrons into fatty acids and glycerol (Goldberg 1996). The products of hydrolysis (fatty acids and glycerol) then diffuse into adipose or muscle cells and are either used for energy or stored for later use (Goldberg 1996). After chylomicrons interact with lipoprotein lipase they circulate back into the main blood stream where they interact with HDL again and donate back apoprotein C. After this process, the chylomicron is considered a chylomicron remnant (Cooper 1997). The chylomicron remnants contain a smaller amount of triglycerides than their precursor but have slightly higher levels of cholesterol esters and fat soluble vitamins (Cooper 1997). Chylomicron remnants are endocytosed by the liver through a receptor-ligand interaction (Cooper 1997). The apoprotein B48 present on the remnant binds to its receptor on the liver cell which causes the liver cell to endocytose the remnant (Cooper 1997). Apoprotein E present on the chylomicron remnant can also interact with LDL receptors on the liver cells and allow for endocytosis (Cooper 1997). The materials that make up the chylomicron remnants are then used to synthesize new lipoproteins, called nascent very low density lipoproteins (VLDLs) (Field 1995). Nascent VLDLs are different from chylomicrons because they contain a lesser amount of triglycerides and a slightly greater amount of cholesterol (Field 1995). Lean cats have an even concentration of both large and medium VLDL particles (Jordan et al 2008). During the synthesis of VLDL apoprotein B_{100} is added. When the synthesis of VLDL particles is complete, they leave the liver cell by exocytosis, and circulate through the capillaries to the main circulation where they receive apoprotein C and apoprotein E from HDL (Bauer 2004). Once they receive those apoproteins from HDL they become mature. When they reach adipose tissue, apoprotein C activates lipoprotein lipase (Bauer 2004). Again, the lipase metabolizes the triglycerides and the

components, i.e. glycerol and fatty acids diffuse into adipose cells where they are either used for energy or stored as triglycerides (Solomon et al 2002). After VLDL has interacted with the lipase, it interacts with HDL to donate back the apoprotein C. This process converts the VLDL to intermediate density lipoprotein (IDL). VLDL remnants can also be metabolized to IDLs via another enzyme called hepatic lipase (Demacker 1988). Hepatic lipase is synthesized in the liver but can be found in the adrenal glands and ovaries, and unlike lipoprotein lipase, hepatic lipase does not need to be activated by an apoprotein (Connelly 1999). Similar to humans cats have a higher level of hepatic lipase than lipoprotein lipase (Demacker 1988). One study found that hepatic lipase may contribute to lipid metabolism after a meal because when the enzyme was inhibited in the cat, there was an increase in plasma triglycerides and phospholipids (Demacker 1988).

IDLs can interact with cells that have LDL receptors which bind to their apoprotein E with high affinity. This results in further hydrolysis of the triglycerides present in IDLs and the lipoprotein now becomes low density lipoprotein (LDL). Also, some IDLs will circulate directly to the liver where they are metabolized by hepatic lipase to LDLs (Connelly 1999). The remaining LDLs continue to circulate through the blood stream binding to LDL receptors via their apoprotein B_{100} and delivering cholesterol to cells that need it. Cells in need of cholesterol to synthesize steroid hormones such as aldosterone, cortisol, or sex hormones, synthesize the LDL receptor and transport it to their surface membrane (Solomon et al 2002). Any remaining LDLs not taken in by other cells are endocytosed by the liver where they are further degraded. In the cat, LDL particles can be separated into large, medium small, and very small particles (Jordan et al 2008). Lean cats have been shown to have mainly large LDL particles (Jordan et al 2008). HDLs are primarily synthesized in the liver and are mainly composed of cholesterol and

small amounts of triglycerides. They are responsible for collecting excess cholesterol from peripheral cells, adding an ester to it using lecithin: cholesterol acyltransferase (LCAT) and transporting it to the liver for degradation (Solomon et al 2002).

Cats have been referred to as high 'HDL animal' because they possess greater concentrations of HDL cholesterol than LDL cholesterol (Ginzinger et al 1999 and Demacker et al 1987). In cats, HDL particles can be separated into large, medium and small particles (Jordan et al 2008). It has been shown that lean cats have a higher concentration of medium HDL particles than small HDL particles (Jordan et al 2008). During the exchange of apoproteins between chylomicrons and HDL, apoprotein AI is transferred from the chylomicron to HDL. The apoprotein AI is responsible for activating LCAT, which synthesizes cholesterol esters from a carboxylate group on fatty acids and a hydroxyl group from cholesterol.

The process of returning excess cholesterol from peripheral cells to the liver is called reverse cholesterol transport (RCT) and cholesteryl ester transfer protein (CETP) is one of the molecules involved in this process (Fielding and Fielding 1995). CETP helps transport cholesterol esters and triglycerides between lipoproteins (Murakami 1995). For example, it transfers triglycerides from VLDL and LDL to HDL in exchange for cholesterol esters and vice versa (Murakami 1995). CETP also exchanges the same molecule between lipoproteins, i.e. exchanging a triglyceride for another triglyceride or a cholesterol ester for another cholesterol ester between lipoproteins. CETP genes are found in liver, spleen, and adipose tissue and their expression is upregulated by the presence of cholesterol (McPherson 2008). When VLDLs and LDLs are being cleared from the circulation by the liver at an efficient rate, CETP transfers cholesterol from HDL to the liver for biliary secretion (McPherson 2008).

Hepatic lipase then hydrolyzes the triglycerides, cholesterol esters and phospholipids from the HDLs and converts HDLs into its different subfractions i.e. HDL2 and HDL3 (Connelly 1999, and Demacker 1988). CETP also transfers cholesterol esters from HDL to the liver through a process called selective uptake, which does not involve HDL degradation (McPherson 2008). A process different from selective uptake is the CETP transfer of cholesterol esters from HDL to LDL. The LDL then binds to LDL receptors on hepatocytes and is endocytosed and degraded. In the liver, the cholesterol can be recycled and secreted with newly secreted lipoproteins or secreted with bile into the intestine and used to synthesize chylomicrons (Fielding and Fielding 1995). In contrast to humans, investigators have been unable to measure CETP activity in the cat (Ginzinger et al 1999 and Watson et al 1995). When CETP activity is absent, cholesterol from HDL cannot be transferred to triglyceride-rich lipoproteins nor can triglycerides be transferred from triglyceride-rich lipoproteins to HDL (Ginzinger et al 1999).

Endogenous Lipid Metabolism

When blood glucose levels are elevated, plasmas insulin concentrations increase concomitantly stimulating the uptake of peripheral glucose and decreasing the catabolism of glycogen reserves. Glucose is normally stored as glycogen in the liver and in muscle tissue through a process called glycogenesis. However, when energy levels are high or too much food has been consumed some of the carbohydrates will get converted into fat and stored. This is called endogenous triglyceride synthesis. The endogenous triglycerides synthesized from glucose in the liver and adipocytes are tripalmitin and tristearate. Endogenous triglycerides synthesized in the liver circulate via VLDLs to adipose tissue, where they bind to lipoprotein lipase and release glycerol and fatty acids which after reesterification are stored as triglycerides for later use.

When glycogen reserves in the liver are filled, glucose enters the pentose phosphate pathway, in order to be converted into fat and stored as energy for later use. The fact that glucose is able to enter the pentose phosphate pathway and not the glycolytic pathway directly is due to the inhibition of isocitrate dehydrogenase by large levels of ATP and NADH produced in the mitochondria. The inhibition of isocitrate dehydrogenase increases the levels of mitochondrial citrate and isocitrate. This causes the citrate to diffuse into the cytosol via the tricarboxylate carrier and inhibit phosphofructokinase-1, thereby inhibiting glucose from entering the glycolytic pathway. The excess citrate in the cytosol is also converted to oxaloacetate and acetyl CoA by the enzyme citrate lyase. Acetyl CoA is needed for the synthesis of fatty acids. Citrate activates acetyl CoA carboxylase, which is an enzyme that regulates fatty acid synthesis. During the pentose phosphate pathway, glucose-6-phosphate dehydrogenase removes two hydrogens from the first carbon of the glucose-6-phosphate molecule to yield NADPH , which is a hydrogen carrier needed for fatty acid synthesis. The pentose phosphate pathway yields glyceraldehyde-3-phosphate which then enters glycolysis to yield acetyl CoA. The acetyl CoA combines with oxaloacetate to form citrate and diffuses back into the cytosol, where it is again broken down into oxaloacetate and acetyl CoA. Acetyl CoA is converted to malonyl CoA by acetyl CoA carboxylase. Malonyl CoA then reacts with acyl carrier protein (ACP) to form malonyl ACP. Malonyl then goes through more series of reactions to yield palmitate and stearate which are fatty acid chains that get added onto glycerol-3-phosphate. After fatty acids and glycerol are synthesized in the liver from glucose they are esterified into triglycerides and packaged into VLDLs to be transported to adipocytes for storage.

Endogenous lipogenesis also occurs in adipocytes. Insulin activates glucose transporter four (GLUT4) which transports glucose into the cell where it is immediately phosphorylated to

glucose-6-phosphate. The glucose-6-phosphate then enters glycolysis and is converted to glycerol 3 phosphate to yield glycerol (backbone of triglyceride) or via pyruvate acetyl Co-A which is needed for the synthesis of fatty acids.

Lipid Metabolism in Starvation

In starvation, when energy levels are low and glycogen reserves are depleted, the triglycerides stored in adipose tissue are hydrolyzed by hormone sensitive lipase.

During times of exercise or stress, the activity of hormone sensitive lipase is regulated by the catecholamines, epinephrine and norepinephrine, as well as insulin-antagonistic hormones such as cortisol and glucagon, among others. Activated hormone sensitive lipase hydrolyzes triglyceride to monoacylglycerol. Monoacylglycerol is then hydrolyzed by monoacylglycerol lipase to form glycerol and fatty acids. These molecules diffuse into the circulation where fatty acids bind to serum albumin and circulate to the liver and skeletal muscles to be used for cellular respiration involving the following pathways: beta-oxidation pathway, the citric acid cycle, and the respiratory chain. The ATP which is produced by the metabolism of fatty acids provides energy for gluconeogenesis. The hydrolyzed glycerol is used in gluconeogenesis to yield glucose. (Salaway 2004)

Beta-Oxidation Pathway

In order for fatty acids to enter the mitochondria and become oxidized, they must first be activated in the cytoplasm to acyl-CoA by acyl-Co A synthetase. Fatty acyl-CoAs are then transported to the mitochondria via the carnitine cycle. Carnitine acyltransferase I is an enzyme located on the outer mitochondrial membrane and is responsible for transferring the acyl group from the cytosol to carnitine to yield acylcarnitine. Acylcarnitine then diffuses into the mitochondrial intermembrane space and is transported to the matrix through the antiporter

carnitine-acylcarnitine translocase located in the inner mitochondrial membrane. The enzyme carnitine acyltransferase II is also located in the inner mitochondrial membrane and transfers the acyl group back to Co A to yield acyl-CoA. The substrate acyl-CoA can be metabolized in the beta-oxidation pathway to yield acetyl coenzyme A. Acetyl CoA synthesized from fatty acids cannot be carboxylated to pyruvate and enter a reversed glycolysis reaction to yield glucose, because the pyruvate dehydrogenase and pyruvate kinase reactions are irreversible. Instead, Acetyl CoA will enter the citric acid cycle to yield three more forms of energy molecules (NADH, $FADH_2$, GTP) that will be used in the electron transport chain to yield ATP in oxidative phosphorylation. (Pelley 2007)

Gluconeogenesis

The carbon skeletons needed for the synthesis of glucose come from glycerol. After triglycerides are hydrolyzed to fatty acids and glycerol, glycerol is phosphorylated in the liver by glycerol kinase to glycerol 3-phosphate. Glycerol 3-phosphate dehydrogenase then reduces the glycerol 3-phosphate to dihydroxyacetone phosphate (DHAP). Dihydroxyacetone phosphate is a three carbon molecule, therefore, two molecules of DHAP are needed to make one molecule of glucose in gluconeogenesis and this process requires the energy from six ATP. (Pelley 2007)

Lipid Metabolism in Obesity

An increase in weight gain is a result of excess triglyceride storage in adipose tissue. If energy intake continues to exceed energy expenditure obesity will ensue (Spiegelman et al 2001). Obesity is a risk factor for diabetes in both humans (Eckel et al 2005) and cats (Scarlett and Donoghue 1998) because it is associated with insulin resistance and glucose intolerance. Obesity is also known to cause alterations in lipid and lipoprotein concentrations in people (Despress 1990) and cats (Jordan et al 2008). Some of the negative effects seen in lipid metabolism due to

obesity are similar between cats and humans and some are different (Hoenig et al 2003). Obese individuals have elevated cholesterol, triglycerides, and apoprotein B, as well as higher levels of VLDLs, LDLs and lower levels of HDLs (Despress 1990). Newly obese cats unlike long-term obese cats and humans have been shown to have increased levels of HDL, which may be a protective mechanism against cardiovascular disease (Hoenig et al 2003). Long term obese cats have higher levels of non-esterified fatty acids, triglycerides, and VLDLs and decreased levels of HDLs but unlike humans, hypertension and atherosclerosis has not been documented in the obese cat (Jordan et al 2008). Although there is a decrease in HDL in obese cats, obese cats still have a higher concentration of HDL than in human subjects. Research has shown that LPL in obese individuals is inhibited in adipose tissue but activated in muscle tissue (Phillips 1996 and Hoenig M, et al 2006). When insulin is unable to activate its receptor, it is unable to activate LPL in adipose tissue and inhibit hormone sensitive lipase. Inhibited LPL can no longer metabolize chylomicrons or VLDLs and activated hormone sensitive lipase continues to mobilize triglycerides from adipose tissue (Salaway 2004). This interferes with the homeostasis of free fatty acids in the plasma and contributes to the high elevated levels of NEFAs seen in obese individuals and in cats. Since these NEFAs are not properly stored in adipose tissue due to insulin resistance, they are stored in the liver and muscle (Saltiel and Kahn 2001). The excess free fatty acids stored in liver and muscle tissue disrupt glycolysis, normal glucose uptake and lipid metabolism (Golay et al 1984). In the liver, free fatty acids are esterified to triglyceride and transported to the blood via VLDLs, causing hyperlipidemia in obese individuals (Salaway 2004). To investigate the digestion and absorption of exogenous triglycerides in people with hyperlipidemia, subjects are given an oral fat load containing vitamin A. Vitamin A is digested and absorbed similar to cholesterol and is transported in the plasma via chylomicrons. Unlike

cholesterol, vitamin A is not recycled back into VLDL and is either bound to retinol-binding protein (RBP) and released into the plasma (Smith et al 1979) or stored in the liver (Blomhoff et al 1982 and 1984). Therefore vitamin A is a good indicator of triglyceride rich lipoproteins of intestinal origin and is often use to trace the catabolism of lipoproteins. Determining postprandial lipoproteins is important because these triglyceride rich proteins are thought to be atherogenic (Karpe et al 1999).

Recent studies have shown that particle size not the concentration of lipoproteins is associated with many of the co-morbidities of obesity. These lipid alterations can cause changes in insulin secretion and insulin action in people but the mechanisms involved in its pathogenesis and progression to diabetes are still unknown (McGarry 2002). In humans elevated levels of large particle VLDLs showed a decrease in insulin sensitivity (Goff et al 2005) and an increase in coronary artery disease (Freedman et al 1998). The small HDL particles present in obese people (Goff et al 2005) result from the presence of elevated levels of triglycerides in HDLs, which leads to the activation of hepatic triglyceride lipase (Murakami et al 1995). Hepatic triglyceride lipase breaks down HDLs into smaller particles and increases their clearance from the body (Murakami et al 1995). The decrease in HDL interferes with the reverse cholesterol transport system and results in less cholesterol being transported from the periphery to the liver for elimination. Excess cholesterol remains in peripheral cells such as macrophages, or can remain in LDLs and be eliminated in the liver via hepatic LDL receptors where it will get excreted as bile. However, this process of elimination from the periphery is not as efficient as reverse cholesterol transport. The decrease in HDL also increases the risk of developing insulin resistance and heart disease in people (Garvey et al 2003). Similar to HDL, increasing the concentration of triglycerides in LDLs increases their affinity for hepatic triglyceride lipase,

which metabolizes them into small dense LDL particles (de Graaf et al 1993). Studies have shown that obese people (Goff et al 2005) and obese cats (Jordan et al 2008) have a greater concentration of small LDL particles and a lower concentration of large LDL particles. In humans, these small LDL particles have been found to be associated with a decrease in insulin sensitivity (Goff et al 2005). They are also more susceptible for oxidation, have less affinity for the LDL receptor, and have a greater affinity for cell surface receptors (de Graaf et al 1993). The smaller oxidized sub LDL particles damage the arterial walls because they can easily penetrate the endothelial lining and are therefore targets for macrophages (Blake et al 2002 and Sparrow et al 1989). The oxidized LDL particles are phagocytosed by macrophages via the scavenger receptor however, macrophages cannot metabolize the lipoprotein, and therefore LDLs accumulate inside the cytoplasm, which leads to atherosclerosis (Berliner 1995). Not only did long term obese cats show an increase in VLDLs and a decrease in HDLs, but more specifically they showed an increase in large and medium sized VLDL particles and the concentration of HDLs was mainly made up of small particles (Jordan et al 2008). Although these findings were similar to those found in obese humans, atherosclerosis and cardiovascular disease has not been detected in the obese cat, suggesting other factors are involved in the disease progression (Jordan et al 2008).

Dietary fat content can also influence lipid metabolism and insulin resistance associated with obesity. Saturated fatty acids (SFAs) increase plasma cholesterol, triglyceride, free fatty acid concentrations and decrease insulin sensitivity in humans (DeFronzo et al 1991) and in cats (Wilkins et al 2004). In contrast, studies have also shown that people who consume polyunsaturated fatty acids (PUFAs) (DeFronzo et al 1991) and cats that are fed PUFAs (Wilkins et al 2004) have improved insulin sensitivity and lipid regulation because plasma triglycerides

and LDL decrease, and the HDL fraction increases. It is believed that the beneficial effects of unsaturated fatty acids are derived from their ability to increase the expression of peroxisome proliferator activated receptor-γ (PPAR-gamma) (Luan et al 2001).

Lipid Peroxidation

In human subjects chronic oxidative stress is associated with obesity and insulin resistance (Evans et al 2003). Oxidative stress occurs when there is an imbalance between free radicals and antioxidants (Madamanchi et al 2005). Lipid peroxidation is a type of oxidative stress where lipids are oxidized into lipid radicals (Porter 1995). Obese individuals show a faster rate of lipid oxidation when compared to lean individuals (Van Gaal et al 1998) because they have elevated lipid pools in adipose tissue and blood (Furukawa et al 2004). Lipid radicals are not stable molecules because they contain unpaired electrons and are therefore highly reactive (Porter 1995). Lipid radicals like other free radicals disrupt normal cellular function and can lead to cell death. The most common lipids that are oxidized are polyunsaturated fatty acids (PUFAs) because they have multiple double bonds and have highly reactive hydrogens (Porter 1995). The double bonds present in PUFAs weaken the carbon-hydrogen bond next to them. This weak carbon-hydrogen interaction makes the hydrogen more susceptible to react with free radicals (Porter 1995).

Lipid peroxidation increases dramatically in the postprandial phase (Musso et al 2006) because VLDL and LDL are enriched with lipids. Lipid peroxidation occurs in three main steps, initiation, propagation, and termination (Bolland 1949 and Batemen 1954). The first step, initiation, occurs when a polyunsaturated fatty acid reacts with a reactive oxygen species (ROS) such as, OH, forming a lipid radical and water (Bolland 1949 and Batemen 1954). The lipid radical then proceeds to the next step, propagation, where it reacts with molecular oxygen to

generate lipid peroxyl-radical (Bolland 1949 and Batemen 1954). This radical reacts with other polyunsaturated fatty acids or lipids in the cell membrane to create lipid hydroperoxide and more lipid radicals (Bolland 1949 and Batemen 1954). This process is a chain reaction because it continues producing lipid radicals that destroy cell membranes resulting in even more lipid radicals. The cell membrane is an extremely important part of the cell because it regulates permeability and contributes to cellular and molecular signaling (Solomon et al 2002). A disrupted membrane can lead to cytotoxicity by inhibiting metabolic processes, as well as, altering ion channels resulting in abnormal cell signaling (Catala 2006). Lipid radicals can also affect the membranes of mitochondria which can result in more ROS production due to electron leakage. The increased production in ROS results in an increase in the synthesis of lipid radicals and the vicious cycle continues.

During propagation, large amounts of lipid hydroperoxide are created (Bolland 1949 and Batemen 1954). Lipid hydroperoxide can react with reducing agents such as Fe++, producing lipid alkoxyl radical (Catala 2006). This molecule like the lipid radicals contributes to the chain reaction and produces more lipid radicals. Lipid hydroperoxide can also react with other reduced metals or ascorbate to produce reactive aldehyde products such as malondialdeyde (MDA) (Catala 2006). MDA is the most abundant aldehyde product from lipid peroxidation and is often used as a biomarker for oxidative stress (Catala 2006). Reactive aldehydes are stable molecules and can therefore sometimes diffuse from one cell to a different part of the body where they exert damaging effects (Catala 2006). Reactive aldehyde products can also act as toxic second messengers in cells and add to the adverse effects of lipid peroxidation by affecting cell signaling and gene transcription (Catala 2006).

Linoleic acid is an essential fatty acid of all mammals for the production of other lipids and cholesterols (Solomon et al 2002). Linoleic acid is a polyunsaturated acid and is therefore commonly oxidized in the lipid peroxidation process (Bolland 1949 and Batemen 1954). It is oxidized at the methylene group (C11) in between the 9,10cis and 12,13cis double bonds (Porter 1995). Once linoleic acid is oxidized to a fatty acid radical, it readily reacts with oxygen to yield a lipid peroxyl radical. The rate of lipid peroxyl radical synthesis depends on the amount of oxygen present and the reaction is very fast. This reaction is reversible and is called beta fragmentation, because the bond between oxygen and the lipid radical is located at the beta position of the radical. Peroxyl radicals can circulate in the body from a few milliseconds to seconds and can therefore react in different pathways (Marnett 1987). If peroxyl radicals do not convert back to lipid radicals, they will react with other polyunsaturated lipids (Bolland 1949 and Batemen 1954). This step in the reaction is the slowest (Porter 1995) and contributes the most to the chain reaction by reacting with cell membranes which are extremely susceptible to damage by lipid radicals because they are made up of polyunsaturated fatty acids.

Lipid radicals are only terminated when they react with another lipid radical or when antioxidants, such as, superoxide dismutase, catalase, and peroxidase reduce the molecule to a stable form (Bolland 1949 and Batemen 1954). Antioxidants convert peroxyl radicals to lipid hydroperoxide, therefore terminating the ability of the peroxyl radical to interact with other lipids and creating more free radicals (Bolland 1949 and Batemen 1954). Dietary antioxidants consist of tocopherols, ascorbate, carotenoids, thiols, polyphenols, and other micronutrients and have been shown to reduce oxidative stress. For example vitamin E donates hydrogens readily to free radicals and still remains stable and un-reactive (Bowry 1999). Human adipocytes exposed to oxidative stress and then treated with antioxidants showed a decrease in ROS and an increase in

proliferation and viability (Roche et al 2009). Consumption of a diet rich in flavonol reduced DNA damage in lymphocytes that were exposed to an oxidative challenge ex vivo (Lean et al 1999). Patients with type 1 and 2 diabetes given a diet supplemented with α-lipoic acid, vitamin E, or selenium for 12 weeks, showed a decrease in lipid peroxidation (Ziegler et al 1996). Although antioxidants are beneficial, they do not react as fast as molecular oxygen or lipid radicals do and therefore if there is an increase in free fatty acids as seen in obesity, then the detrimental effects of lipid peroxidation outweigh the beneficial effects of antioxidants. Research has also shown conflicting data on the debate of potential health benefits of antioxidants. The number of assays available on the market for antioxidants and indicators of oxidative stress is limited and they often lack specificity and are poorly validated (Hermans et al 2007). Diabetic and non diabetic cats fed Purina Dietetic Management (DM) cat food containing high levels of vitamin E for 8 weeks did not show a difference in MDA or in reduced or oxidized glutathione (GSH:GSSG) before or after the study (Webb 2009). Another study looking at oxidative stress and kidney failure found that cats with renal insufficiency had higher levels of MDA compared to healthy cats however, even after supplementation with antioxidants for four weeks, the MDA levels remained unaltered (Yu and Paetau-Robinson 2006).

Glucose Metabolism in Lean Subjects

Postprandially, when glucose levels are high, glucose enters pancreatic beta cells via glucose transporter two (GLUT2). GLUT2 has the highest Km for glucose of all the glucose transporters. In the beta cell the glucose undergoes glycolysis and is metabolized to pyruvate. Pyruvate is converted to acetyl co A and enters the citric acid cycle (CAC) to yield the electron carrying molecules NADH, $FADH_2$, and GTP. These reduced molecules donate their electrons to the electron transport chain (ETC) resulting in oxidative phosphorylation and the synthesis of

many ATP molecules. ATP binds to ATP-dependent K channels located in the beta cell membrane, causing them to close and resulting in depolarization of the beta cell. The depolarization opens voltage-gated calcium channels allowing calcium to enter into the cell. The increase in intracellular calcium concentration activates the enzyme phospholipase C which cleaves phosphatidyl inositol 4, 5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to receptor proteins in the endoplasmic reticulum, allowing for release of calcium from the ER via IP3 gated channels. The influx of calcium stimulates secretory vesicles to fuse with the cell membrane, and release insulin into the extracellular fluid.

Insulin binds to its receptor on different tissues and activates insulin-receptor substrate (IRS), inducing an intracellular signaling cascade and cellular effects. The primary action of insulin is to stimulate glucose uptake in insulin sensitive tissues by increasing the movement of glucose transporters to the cell surface. The main glucose transporters involved in peripheral glucose uptake into tissues are GLUT4 and GLUT1. GLUT1 is insulin-insensitive and is located in the plasma membrane. It has a low km and operates primarily at basal glucose concentrations. GLUT1 can be found in most tissues, including, brain, red blood cells, blood brain barrier, as well as muscle and adipose tissue. The insulin-sensitive GLUT4, however, responds to insulin by translocating to the cell membrane where they increase glucose uptake 10-40 fold (Hashiramoto and James 2000). Insulin sensitive GLUT4 is the primary transporter found in muscle and adipose tissue.

In adipose and muscle tissue, glucose enters the glycolytic pathway and CAC and ETC to yield energy molecules. In adipose tissue, glucose can also be converted to fatty acids and stored as triglycerides or in muscle tissue glucose can be stored as glycogen. When glucose levels are low, the hormone glucagon is released from alpha cells in the pancreas which stimulates the liver

to break down glycogen and release glucose molecules into the circulation. After glycogen reserves have been depleted, the liver synthesizes glucose from non-carbohydrates through gluconeogenesis.

Glucose Metabolism in Obese Subjects

As animals and people become obese, insulin becomes less effective in its ability to stimulate glucose uptake in cells of the liver, skeletal muscle and adipose tissue. In people, insulin also becomes less effective in suppressing hepatic glucose output (Defronzo 1991; Porte 1991; Paquot et al 2002). The response to this defect is an increase in the output of insulin which results in hyperinsulinemia. A 50% increase in body mass index (BMI) in cats causes about a 60% increase in insulin resistance (Hoenig et al 2006). Insulin resistance has been well documented in the cat (Hoenig et al 2007; Wilkins et al 2004); however obese cats usually show fasting euglycemia. This is explained by the fact that obese cats maintain hepatic insulin sensitivity despite peripheral insulin resistance as shown in a recent study by Kley et al (2009). In contrast, obese humans with high insulin levels showed an increase in endogenous glucose production, indicating hepatic insulin resistance (Weyer et al 1998).

Research has shown that in the beginning stages of type 2 diabetes glucose transporter activity is impaired in muscle tissue and this has also been shown in individuals who have altered lipid metabolism (Rothman et al 1995). In the normal state, insulin stimulates glycogen synthesis and increases glucokinase activity. When insulin action is impaired, glycogen synthesis is decreased and glycogenolysis is activated resulting in the release of glucose from the liver thus contributing to the hyperglycemic state. Due to hyperglycemia, GLUT2 gene expression is increased in islets and liver (Tiedge et al 1991). In muscle tissue a decrease in insulin sensitivity, leads to an increase in fatty acid oxidation in order to synthesize energy yielding molecules. The products

from beta-oxidation (acetyl CoA and NADH) inhibit pyruvate dehydrogenase, an enzyme that regulates glycolytic metabolites into the Krebs cycle. In the absence of activated pyruvate dehydrogenase, the glycolytic metabolites are converted into lactate and alanine and metabolized in the liver during gluconeogenesis. Therefore, fatty acid oxidation in muscle tissue contributes to the hyperglycemia seen in obese individuals. (Salaway 2004) In adipose tissue the down regulation in GLUT4 decreases the activity of glycolysis and decreases the synthesis of glycerol 3-phosphate. Without the formation of glycerol 3-phosphate, there is no other molecule available for the re-esterification of the fatty acids formed by hormone sensitive lipase. As a result, the free fatty acids diffuse into the blood circulation. (Salaway 2004) Obesity in cats is also characterized by a decrease in the activity and expression of lipoprotein lipase in adipose tissue, contributing to the increase in free fatty acids in blood (Hoenig et al 2006). Obese cats have low GLUT4 expression in both muscle and adipose tissue, whereas the expression of GLUT1 is not different between lean and obese cats (Brennan et al 2004). The onset of this reduction is early and arises before any fasting hyperglycemia is evident (Brennan et al 2004). It is hypothesized that this impairment in GLUT4 transporter is due to a defect in the transport vesicle responsible for delivering the transporter to the cell membrane (Brennan et al 2004). The reduced expression of GLUT4 in obese cats could serve as an early marker for the progression to diabetes. Other studies have shown that GLUT1 transporter is insulin insensitive because the transporter is already localized in the cell membrane and therefore no significant change was seen in the expression of GLUT1 in obese cats (Hansen et al 1998 and Brennan et al 2004).

Inflammatory Mediators, Cytokines, and Lipid Metabolism

The immune and endocrine systems are linked in many ways with (Wellen and Hotamisligil 2005). For example, the inflammatory response initiated by the immune system depends on

energy metabolized from lipids to fight off diseases, and therefore it activates catabolic pathways and inactivates anabolic pathways such as, the insulin signaling pathway (Wellen and Hotamisligil 2005). The two systems are also connected because the primary cells involved in both systems, i.e. adipocytes and macrophages, function in similar ways and respond to the same hormones, cytokines, and other signaling proteins. Like adipocytes, macrophages can store lipids; however, they become foam cells that lead to atherosclerosis (Wellen and Hotamisligil 2005). When circulating in the appropriate environment, preadipocytes can be phagocytic, display antimicrobial properties, and differentiate into macrophages (Cousin et al 1999). These cells also synthesize the same proteins, such as, PPARgamma, TNF-alpha, IL-6, and matrix metalloproteinases (MMPs) (Wellen and Hotmisligil 2004 and Hotmisligil et al 1993).

A metabolic imbalance such as obesity will result in imbalance in the immune system and lead to inflammatory diseases (Wellen and Hotmisligil 2004). Recent findings have shown that obesity is characterized by chronic low level inflammation in adipose tissue (Xu et al 2003), possibly resulting from the accumulation of macrophages in adipose tissue (Weisberg et al 2003). It is hypothesized that macrophages stimulate the synthesis of inflammatory mediators either alone or synergistic with adipocytes, and macrophages may be an important factor in the development of insulin resistance (Wellen and Hotmisligil 2004). Similar to macrophages, metabolic stresses in obesity, such as endoplasmic reticulum (ER) stress and increased ROS production, can activate inflammatory signaling pathways and contribute to insulin resistance (Ozcan et al 2004 and Lin et al 2005). Inflammatory mediators disrupt insulin signaling by inhibiting the insulin signaling cascade (Wellen and Hotmisligil 2004). Some of these inflammatory mediators include the cytokines IL-6 and IL-1, and the adipokines adiponectin, leptin, and tumor necrosis factor (TNFalpha). It has been shown that short term obesity and

dietary fatty acid composition does not affect immune function in cats (Jaso-Friedmann et al 2008).

IL-6 and IL-1

IL-6 is a cytokine with many functions and is involved in immune and inflammatory responses (Cahill and Rogers 2008). This cytokine can be synthesized by different cells including, adipocytes, activated leukocytes, fibroblasts, and endothelial cells. Research has shown that 30% of circulating IL-6 is synthesized from adipocytes (Mohamed-Ali et al 1997) and this percentage increases with obesity and decreases with weight loss (Mohamed-Ali et al 1997). The increase in IL-6 in obesity may help contribute to the state of chronic, low-grade inflammation. IL-6 secreted from adipocytes stimulates hepatic triglyceride secretion and induces hepatic gluconeogenesis contributing to the hyperglycemic state seen in obesity related type 2 diabetes. Furthermore, IL-6 decreases insulin sensitivity and lipoprotein lipase activity in adipocytes and increases lipolysis and the synthesis of leptin. These findings indicate that IL-6 may play a role in the pathogenesis of insulin resistance in obesity (Lazar 2005). In contrast, *in vitro* experiments have revealed that IL-6 increases GLUT4 translocation, glucose uptake, and fat oxidation in muscle and fat cells (Carey et al 2006). Research has also shown that when humans were infused with IL-6 during a hyperinsulinemic-euglycaemic clamp they had enhanced insulin action (Carey et al 2006). Therefore, IL-6 may not always be one of the culprits involved in type 2 diabetes.

IL-1 is one of the primary proinflammatory cytokines involved in an immune response and induces fever, lymphocyte activation, and acute phase protein synthesis (Stylianou and Saklatvala 1998). It is synthesized in many immune cells including, macrophages, dendritic cells, B cells, and T cells (Dinarello 1991). IL-1 increases during hyperglycemia, regulates

insulin secretion, is involved in central leptin action (Wellen and Hotamisligil 2005), and induces expression of IL-6 (Cahill and Rogers 2008). IL-1 receptor antagonist is a protein that competes with IL-1 for the same binding site on the IL-receptor and therefore blocks the effects of IL-1 (Dinarello 1998). IL-1 receptor antagonist is synthesized in human white adipose tissue and is upregulated in obese individuals (Juge-Aubry et al 2003). Mice administered IL-1 receptor antagonist showed a decrease in the effectiveness of leptin on food intake and body temperature (Luheshi et al 1996). Research has also shown that mice lacking the IL-1 receptor had increased body weight and food intake and showed decreased leptin sensitivity, fat utilization, and locomotor activity when compared to wild-type mice (Garcia et al 2006). These studies indicate that some of the actions of leptin on food intake and body temperature are mediated by IL-1, however, in obese individuals the leptin receptor becomes defective.

TNF alpha

TNF-alpha is a proinflammatory cytokine that is released from macrophages, mast cells, neuronal cells, fibroblasts, and adipocytes. It acts by inducing hepatic C-reactive protein synthesis and IL-6 expression (Hotamisligil et al 1993). TNF-alpha is also known to cause the release of monocyte-chemoattractant protein-1 (MCP-1) from preadipocytes. MCP-1 attracts macrophages to adipose tissue, which results in the release of more inflammatory mediators and contributes to insulin resistance (Wellen and Hotamisligil 2005). TNF-alpha was the first cytokine discovered to show a link between inflammation and obesity (Hotamisligil et al 1993). Investigators found that this cytokine was over-expressed in the adipose tissue of obese rodents (Sethi and Hotamisligil 1999), obese cats (Hoenig et al 2006), and obese humans (Hotamisligil et al 1993). Furthermore, weight loss in obese humans leads to a decrease in expression of adipose TNF-alpha (Hotamisligil et al 1995). TNF-alpha contributes to insulin resistance by inhibiting

insulin receptor substrate through serine phosphorylation (Hotamisligil et al 1996). Serine phosphorylation decreases the interaction between insulin receptor and IRS-1 and decreases tyrosine phosphorylation in response to insulin (Hotamisligil et al 1996). TNF-alpha also inhibits the effects of lipoprotein lipase,contributing to the increase in free fatty acids in obesity.

Chapter 3 Materials and Methods

Animals and Diets

Twenty lean and neutered domestic shorthair cats (10 males and 10 females) were used in this study. Cats were maintained at the University Of Illinois College Of Veterinary Medicine Animal Care Facility under standard colony conditions. They were housed individually in cages and were given free access to water. Animal studies were approved by the University of Illinois 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. Cats were determined healthy based on the results of physical examination and clinical laboratory data. All cats were socialized daily and were accustomed to daily handling.

Prior to the beginning of this study all cats had been fed a commercial diet (Purina ProPlan, Nestle Purina, St. Louis, MO) for 8 months. The cats were equally and randomly assigned to one of two food groups, control diet C (10 cats; 5 males and 5 females) or diet AOX (10 cats; 5 males and 5 females). The composition of the diets is listed in Table 1. Food intake was measured daily, and the cats were weighed once weekly.

Study Design

Time 0 refers to the time of experiments when all cats were lean; time 10, 30, and 60 refers to the time of experiments when cats had increased their body weight by 10, 30, and 60%, respectively. The increase in weight was achieved by allowing increased food intake. Indirect calorimetry measurements were performed on all cats one week prior to other testing. An intravenous glucose tolerance test (IVGTT) was performed one week after indirect calorimetry measurements were performed. The cats were allowed to rest for one day and on day 3, Mazola

oil containing vitamin A was given orally by syringe and blood samples were taken at various intervals for 6 hours. Nuclear magnetic resonance imaging was performed within one week after completion of the lipid challenge test.

Sample Processing

To allow for blood sampling, intravenous catheters were placed into the jugular vein after tranquilization of the cats with 1-2 mg/kg telazol subcutaneously. The catheters were placed 15-18 hours before each testing period. Catheter patency was maintained by injection of 0.5ml of 0.38% sterile citrate flush (citric acid, trisodium salt dehydrate, Sigma Co., MO) every 8-14 hours. All blood samples were collected through the jugular catheter into tubes containing EDTA. The blood was placed on ice immediately and centrifuged at 405 x *g* for 15 minutes. The plasma was stored at -80°C until assayed. For measurements of catalase, glutathione peroxidase, and superoxide dismutase the red blood cells (RBC's) were washed four times with 0.9% NaCl and they were lysed with cold double distilled water as described by the manufacturer (Cayman Chemical Ann Arbor, MI) .

Indirect Calorimetry

The width of the Plexiglas calorimetry chambers was 20 in. and the height was 16 in. The chamber operated at a flow of 5 liters per min. A silica drying column was used to equilibrate the humidity. Respiratory exchange ratio, heat, and flow of air through the calorimetry chamber (liters per minute) were measured using an open-circuit Oxymax System (Software, version 5.0, Columbus Instruments, Columbus, OH). System settle time for each chamber was equal to 60 s, and system measure time for each chamber was equal to 30 s. Calibration of the calorimeter chambers was performed daily using standard gas mixtures against known calibration gas standards. Room temperature was maintained at 25±1 ◦C. The cats were fasted overnight and

indirect calorimetry was performed for 2 h to determine baseline RER and heat production in conscious cats.

The following calculations were used:

RER = $\underline{\text{liters}CO_2 \text{ produced/ liters } O_2 \text{ consumed}}$

Heat production (kcal) = $3.82 \times \text{liters}O_2$ consumed + $1.15 \times \text{liters}CO_2$ produced.

Heat/metablic body size (HMBS) = heat production (kcal/kg)/(body weight)$^{0.75}$

Liters O_2 consumed was determined from the accumulated O_2 at a given time minus O_2 consumed at time 0. Liters CO_2 consumed was determined from the accumulated CO_2 at a given time minus CO2 consumed at time 0.

Intravenous Glucose Tolerance Test (IVGTT)

Intravenous glucose tolerance tests were performed in all cats as follows. Food was withheld for 12 to 14 hours before testing. Two baseline samples were collected for measurement of insulin, and glucose. These 2 baseline samples were averaged. A glucose bolus (50% dextrose, w/v) of 0.5g/kg body weight was then injected and blood samples were taken at 5, 10, 15, 30, 45, 60, 90, 120, 180 min for the measurements of glucose and insulin.

Postprandial Analysis of Lipoproteins

In order to analyze the oxidative modification of lipoproteins postprandially, cats were given Mazola oil (1.5ml/kg) containing vitamin A (100 I. U./kg body weight) orally by syringe. Vitamin A served as a marker for intestinally derived chylomicron particles. Blood samples were taken before and 30, 60, 120, 180, and 360 minutes after oral administration of Mazola oil. Non-esterified fatty acids, cholesterol, triglycerides, and HDL were measured following fractionation by fast protein liquid chromatography (FPLC) (Innis-Whitehouse et al 1998).

Susceptibility of VLDL and LDL to oxidative modification were assessed by monitoring the continuous formation of conjugated dienes (Esterbauer et al 1991). Indices of oxidative stress were assessed by measuring asymmetric dimethylarginine (ADMA), an endogenous competitive nitric oxide synthase inhibitor, with high performance liquid chromatography (HPLC) and nitrotyrosinated-protein was measured by ELISA (Cell Sciences, Canton, MA). These tests were preformed in the laboratory of Dr. Anh Le at Emory University and results were not part of this master's thesis.

Enzyme Assays

The following assays were performed on samples taken at baseline during the IVGTT: The activity of catalase and glutathione peroxidase was measured using commercially available kits from Cayman Chemical (Ann Arbor, Michigan). The glutathione peroxidase samples were diluted 1:200 with sample buffer from the kit prior to assay. The catalase samples were diluted 1:2000 with sample buffer from the kit prior to assay. After the necessary dilutions, all assays were performed following the manufacturer's directions. The activity of superoxide dismutase (SOD) in lysed RBC's was measured using a commercially available kit from RANDOX (Oceanside, CA). The samples were diluted 1:200 using RANSOD diluent prior to assay. For the analysis of superoxide dismutase in the plasma, an intravenous injection of heparin (100 units/kg body weight) was given to each cat and blood samples were taken before and 2, 10, and 15 minutes post heparin injection. Heparin released superoxide dismutase was measured using a commercially available kit from Cayman Chemical (Ann Arbor, Michigan). All enzyme assays were assayed within 4 weeks of sample collection. The recovery from RBC's and plasma was assessed by the addition of known quantities of standard to plasma samples. All samples were tested in duplicate. The standard curve for serial dilutions of plasma from cats was observed to be

parallel to the standard curve provided by the manufacturer for all assays. Addition of 4 concentrations of the standards provided in the catalase kit to feline plasma resulted in mean ± SE recovery of 97 ± 1.1%. The assay had a working range of 2-34nmol/min/ml. Addition of 5 concentrations of the standards provided in the SOD (RBC) kit to feline plasma resulted in mean ± SE recovery of 91± 0.5%. The assay had a working range of 0.168-4.68 units/ml. Addition of 5 concentrations of the standards provided in the SOD (plasma) kit to feline plasma resulted in the mean ± SE recovery of 94± 1.4%. The assay had a working range of 0.025-0.25 units/ml.

Inflammatory Cytokines and Adipokines

IL-6, IL-1, and TNF alpha were also measured in baseline samples from the IVGTT following manufacturer instructions for feline ELISA kits using feline standards (R and D systems Minneapolis, MN).

Other Assays Glucose measurements were performed using colorimetric glucose oxidase method (Genzyme Diagnostics, Charlottetown, PE Canada). Insulin measurements were performed as validated and described previously (Hoenig and Ferguson, 1989)

Magnetic Resonance Imaging

Fat mass was determined with nuclear magnetic resonance as previously described (Hoenig et al 2007). MRI measurements were performed in all cats after injection of tiletamine HCL and zolazepam HCL (2-4mg/kg subcutaneously, Fort Dodge Animal Health, Fort Dodge, IA). All cats were investigated on a Siemens Magnetom Trio 3T horizontal spectroscopy and imaging system with a 16 X 16-cm field of view. Magnetic resonance imaging was performed at 0, 10, 30, and 60% body weight increase. The slice thickness was 3mm with 0.3125 X 0.3125 points. Repetition time (Tr) was 0.4 s, and echo time (TE) was 9.4 ms. The data of the total fat mass

were analyzed with the Amira 5.3 program from Visage Imaging (San Diego, CA). Only slices where both kidneys were visible were analyzed.

Statistical Analysis

The statistical model-based data analysis was carried out using a so-called non-linear mixed effects (NLME) population approach, which consists of fitting all experimental data collected in all cats simultaneously while considering the possible effect of covariates, such as time of study, gender, diet, BW, and measured factors associable to insulin resistance. The NLME method is the most dependable statistical approach, especially in small cohorts, to characterize time functions of repeated measurements in terms of population average model parameters and between-animal variability that can be in part random and in part predictable through covariates. The significance level required for including a covariate in the population model was, in general, P=0.05. A repeated measures analysis of variance was used to compare parameters between groups and time-points to account for repeated measurements over time of the same individuals and an unstructured covariance structure was assumed (p-value of < 0.05).

Chapter 4 Results

Body Weight, BMI, Girth, Energy Intake, and Total Fat

The weight, BMI, girth, energy intake, and total fat of the cats are shown in Table 2. There were no gender or diet differences and all parameters were significantly lower in cats at 0% body weight increase compared to cats at 60% body weight increase ($p < 0.0001$). Total fat was highly correlated with BMI (Figure. 2A), body weight (Figure. 2B), and girth (Figure. 2C). There was also a significant correlation between BMI and body weight (Figure. 3A) and girth (Figure 3B) and between girth and body weight (Figure. 3C). Food intake correlated significantly with BMI (Figure. 4A), body weight (Figure. 4B), and girth (Figure. 4C).

Glucose

The results for baseline (mg/dl) and area under the curve (AUC; g/dl per 180min) concentrations of plasma glucose are shown in Table 3. There was no significant difference between the baseline glucose concentrations of cats fed C or AOX. The mean (±SEM) baseline concentration of glucose in cats at 0% body weight increase was (89.2 ± 3.5) and in cats at 60% body weight increase was (87.4 ± 2.2). The total AUC for glucose was significantly higher in cats fed AOX at 60% (25.6 ± 1.2) at 30% (23.6 ± 0.5) body weight increase ($p < 0.0331$).

Insulin

The insulin secretion pattern in response to glucose is shown in Figure 5. Diet had no effect on insulin concentrations. Baseline insulin concentrations (pmol/L) increased significantly with increasing body weight (Table 4; $p < 0.024$ for all) and was highly correlated with BMI (Figure. 6A), body weight (Figure. 6B), and girth (Figure. 6C). The total AUC for insulin also significantly increased with increasing body weight ($p < 0.001$). The insulin concentrations were significantly different from baseline at 120 and 180 min (Table 4; $p < 0.025$ for both). Insulin

concentrations at 120 and 180 min after glucose administration increased significantly with increasing body weight (Table 4; $p < 0.043$ for all). The total AUC for insulin was highly correlated with BMI (Figure. 7A), body weight (Figure. 7B), and girth (Figure. 7C).

Lipid Analysis

Diet had no effect on plasma baseline cholesterol, triglyceride, HDL, and NEFA. Baseline cholesterol and total AUC for cholesterol and HDL did not change significantly with increasing body weight. An increase in body weight significantly increased triglyceride concentrations ($p < 0.023$ for all). A significant difference was also seen in NEFA concentration when body weight increased to 30 and 60% compared to 0% (Table 5).

Enzyme Assays

The results for catalase, glutathione peroxidase, and superoxide dismutase activity in red blood cells (RBCs) are shown in Table 6. Diet had no effect on enzyme activity and there was no significant difference among the cats with increasing body weights. The results for baseline and AUC heparin-releasable superoxide dismutase (SOD) activity are shown in Table 7. There was no effect of diet on heparin-releasable SOD and there was no significant difference among the cats with increasing body weight.

Cytokine and Adipokine Assays

The results for plasma IL-6, IL-1, and TNF-alpha are shown in Table 8. Diet had no effect on cytokine or adipokine concentrations and there was no significant difference among the cats with increasing body weight.

Indirect Calorimetry

Diet did not have an effect on the baseline respiratory exchange ratio (RER) or on baseline heat production in cats and the results were combined. The RER was significantly higher at the 30

and 60% body weight increase (Table 9.) Baseline heat production in cats increased significantly with increasing body weight.

Table 1: Composition of Diets

	Control (C)	Antioxidants (AOX)
	Nutrient Composition g/100g	Nutrient Composition g/100g
Moisture	7.20	6.56
Protein	45.20	48.10
Fat	18.3	18.5
Carbohydrate	21.13	18.42
Crude fiber	0.71	0.813
Ash	7.46	7.61
Metabolizable energy, kcal/g	3.9	3.87
	Components %	Components %
PFC _ 2061 _R91	2.0	2.0
Soy_Protein Isolate _Bagged	5.325	5.325
L_Lysine	0.033	0.033
Taurine	0.083	0.083
Potassium Chloride	0.610	0.630
Wheat Gluten Vital Bulk	8.033	12.867
Corn Gluten Meal 60	16.639	16.637
Vit Super Prem	0.050	0.050
Wheat Flour Second Clear or Better	17.192	12.867
B_Carotene 10_Blend BASF		0.133
Chicken Whole Carcass and Parts	18.276	18.273
Fish Meal	2.662	2.662
Vitamin E 50	0.020	0.033
Poultry By Product Meal	14.643	14.641
Calcium Carbonate	0.333	0.333
Phosphoric Acid	0.466	0.466
Salt	0.067	0.067
Mineral PX	0.166	0.166
Tallow Edible with VIT E	13.167	13.166
Choline Chloride Liquid	0.236	0.244
Pyridoxine HCL PF		0.012
Astaxanthin Fuji Powder 2% (ASTAREAL PZ AF)		0.012

Table 2. Mean (±SEM) values for body weight (kg), body mass index (kg/ m²), girth (cm), energy intake (kcal/kg), and total fat (%) in 20 cats (n=20, 10 males and 10 females) at different % body weight increases. There was no difference between the two diet groups and the results were combined. Values with the same superscript letter differ significantly (all $p < 0.001$).

	Weight (kg)	BMI (kg/m²)	Girth (cm)	Energy intake (kcal/kg)	Total Fat %
0%	3.4 ± 0.2^{a}	32.4 ± 1.1^{d}	30.2 ± 0.7^{g}	40.6 ± 1.1^{j}	$25\pm1^{m,n}$
10%	$3.9\pm0.2^{a,b}$	$36.9\pm1.3^{d,e}$	$35.3\pm1^{g,h}$	$52.9\pm1^{j,k}$	
30%	$4.4\pm0.2^{b,c}$	$42.4\pm1.5^{e,f}$	$38.6\pm0.8^{h,i}$	$59.7\pm1.1^{k,l}$	$45\pm2^{m,o}$
60%	5.7 ± 0.3^{c}	54.7 ± 2.1^{f}	45.2 ± 0.8^{i}	64.4 ± 1.8^{l}	$57\pm2^{n,o}$

Table 3. Mean (±SEM) baseline (mg/dL) and AUC (g/dL per 180min) concentrations of glucose in 20 cats (n=20, 10 males and 10 females) at different % body weight increases. Values with the same superscript differ significantly ([a] $p < 0.0331$).

	0%	10%	30%	60%
Baseline	89.2±3.5	88.7±1.7	88.8±1.4	87.4±2.2
Diet C AUC	23±0.8	22.7±0.6	23.9±1.1	24.7±0.7
Diet AOX AUC	21.2±1.5	23±0.6	23.6±0.5[a]	25.6±1.2[a]

Table 4. Mean (±SEM) baseline (pmol/L) and AUC (nmol/L per 180min) concentrations of insulin in 20 cats (n=20, 10 males and 10 females) at different % body weight increases. The insulin secretion pattern did not differ significantly between the two diet groups and the results were combined. Values with the same superscript letter differ significantly (^{a,b,c,d,e,f} $p < 0.024$), (^{g,h,i,j,k,l} $p < 0.001$), (^{m,n,o,p} $p < 0.025$), (^{q,r,s,t,u} $p < 0.043$)

	0%	10%	30%	60%
Baseline	31.8±4.9^{a,b,c,m}	58.1±7.8^{a,d,e}	85.7±12.6^{b,d,f,n,o}	111.9±17.1^{c,e,f,p}
120min concentration	31.8±3.6^{q}	43.4±5.1^{q}	55.2±6.6^{n,t}	93.1±17^{t}
180min concentration	25.3±2.5^{m,r}	39.3±4.6^{r,s}	49.4±6.6^{o,s,u}	80.6±10.5^{p,u}
AUC	14.9±1.2^{g,h,i}	17.2±1.7^{g,j,k}	21.1±2.4^{h,j,l}	28.5±3.7^{i,k,l}

Table 5. Mean (±SEM) baseline (mg/dL) and AUC concentrations (g/dL per 360min) of cholesterol, triglyceride, and HDL, and baseline non-esterified fatty acids (NEFA; mEq/L) in 20 cats (n=20, 10 males and 10 females) at different % body weight increases. The cholesterol, triglyceride, and HDL concentrations were not significantly different between the two diet groups and the results were combined. Values with the same superscript letter differ significantly (all $p < 0.023$).

	0%	10%	30%	60%
Baseline Cholesterol	150±10	140±7	142±9	135±9
AUC Cholesterol	52.8±3.7	47.2±2.5	49.9±3.4	47.8±3
Baseline Triglyceride	$18±2^{a,b,c}$	$33±4^{a,d}$	$44±10^{b}$	$67±12^{c,d}$
AUC HDL	44.8±1.4	40.1±1.8	43±1.7	41.2±1.7
NEFA	$0.63+0.05^{e,f}$	0.72+0.10	$0.85+0.04^{e}$	$0.88+0.08^{f}$

Table 6. Mean (±SEM) catalase (Units/mg Hb), glutathione peroxidase (Gpx) (Units/mg Hb) and red blood cell superoxide dismutase (SOD RBC) activity (Units/g Hb) in 20 cats (n=20, 10 males and 10 females) at different % body weight increases. The enzyme activities were not significantly different between the two diet groups and the results were combined. There was no significant difference among the cats with increasing body weight.

	0%	10%	30%	60%
Catalase	210±29	219±26	177±20	187±28
Gpx	97±5	102±5	99±3	97±4
SOD RBC	3124±394	4332±263	2231±191	2735±360

Table 7. Mean (±SEM) baseline (Units/ml) and AUC (Units/ml per 15min) concentrations of heparin releasable superoxide dismutase (SOD) activity in 20 cats (n=20, 10 males and 10 females) at different % body weight increases. The baseline and AUC concentrations of plasma SOD were not significantly different between the two diet groups and the results were combined. There was no significant difference among the cats with increasing body weight.

	0%	10%	30%	60%
Baseline	5.8±0.9	5.7±0.6	5.8±1.1	6±1.1
AUC	3779±875	1722±319	2305±465	3731±1056

Table 8. Mean (±SEM) values for IL-6 (ng/ml), IL-1 (ng/ml), and TNF-Alpha (ng/ml) concentrations in 20 cats (n=20, 10 males and 10 females) at different % body weight increases. The IL-1, IL-6, and TNF-Alpha concentrations were not significantly different between the two diet groups and the results were combined. There was no significant difference among the cats with increasing body weight.

	0%	10%	30%	60%
IL-6	2822±981	2022±544	2111±452	2593±597
IL-1	2689±835	2686±839	2318±632	2325±710
TNF-Alpha	1390±281	1364±328	1353±303	1237±211

Table 9. Mean (±SEM) values for baseline respiratory exchange ratio and heat production (kcal/$kg^{0.75}$) per 24 h in 20 cats (n=20, 10 males and 10 females) at different % body weight increases. Values with the same superscript letter differ significantly (^{a} $p < 0.008$), (^{b} $p < 0.002$), (^{d} $p < 0.0237$), (^{e} $p < 0.0001$), (^{f} $p < 0.0001$), (^{g} $p < 0.0006$), (^{h} $p < 0.001$), and (^{i} $p < 0.0070$).

	0%	10%	30%	60%
RER	$0.7698 \pm 0.006^{a,b}$	0.7832 ± 0.005	0.7989 ± 0.003^{a}	0.7903 ± 0.006^{b}
Heat	$54.7 \pm 1.7^{d,e,f}$	$59.9 \pm 1.2^{d,g,h}$	$66.7 \pm 1.3^{e,g,i}$	$70.7 \pm 1.9^{f,h,i}$

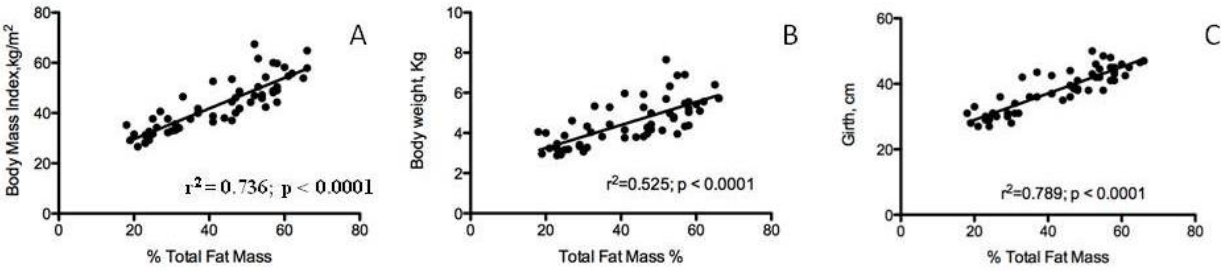

Figure 2. Relationship between total fat mass and body mass index (BMI); A, body weight; B and girth; C in 20 cats (n=20, 10 males and 10 females) at 0% and after 10, 30, and 60% body weight increase.

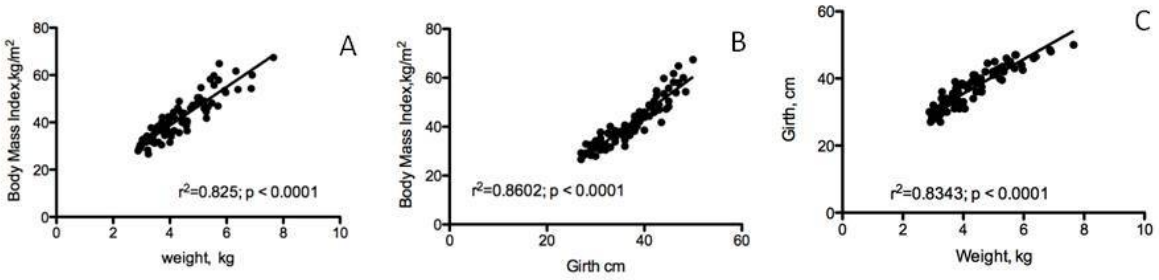

Figure 3. Relationship between body mass index (BMI) and body weight; A, and girth; B and relationship between girth and body weight; C in 20 cats (n=20, 10 males and 10 females) at 0% and after 10, 30, and 60% body weight increase.

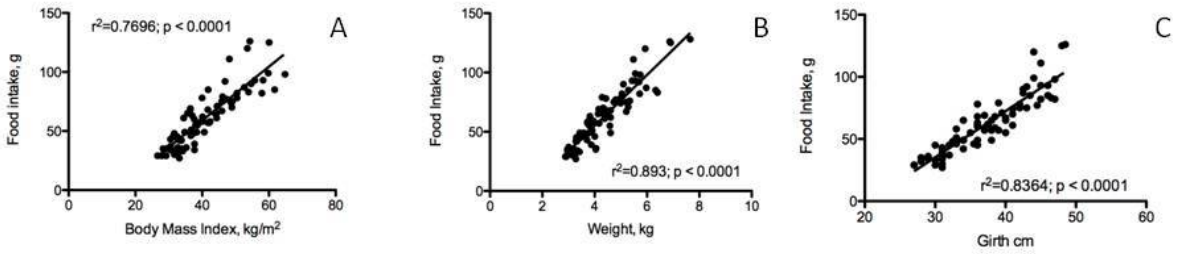

Figure 4. Relationship between food intake and body mass index (BMI); A, body weight; B and girth; C in 20 cats (n=20, 10 males and 10 females) at 0% and after 10, 30, and 60% body weight increase.

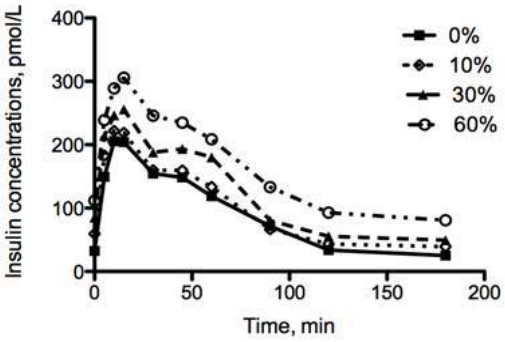

Figure 5. Insulin secretion pattern following a bolus of glucose in 20 cats (n=20, 10 males and 10 females) at 0% and after 10, 30, and 60% body weight increase.

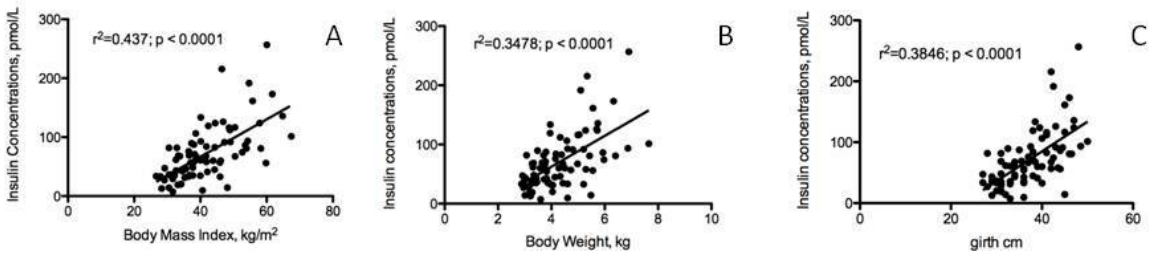

Figure 6. Relationship between baseline insulin concentrations and body mass index (BMI); A, body weight; B and girth; C in 20 cats (n=20, 10 males and 10 females) at 0% and after 10, 30, and 60% body weight increase.

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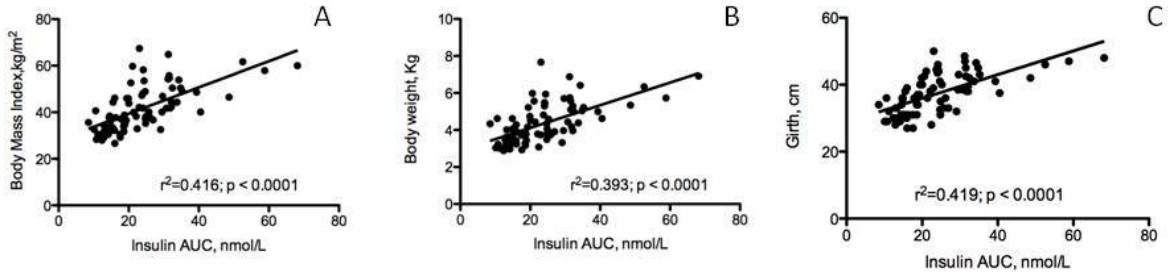

Figure 7. Relationship between insulin area under the curve (AUC) and body mass index (BMI); A, body weight; B and girth; C in 20 cats (n=20, 10 males and 10 females) at 0% and after 10, 30, and 60% body weight increase.

Chapter 5 Summary and Conclusions

The purpose of this research was to examine if an increase in fat mass was associated with an increased inflammatory response and oxidative stress, leading to insulin resistance and abnormal insulin secretion. The effect of antioxidants on oxidative stress was also assessed with increasing fat mass.

Body Weight, BMI, Girth, Energy Intake, and Total Fat

As % body weight increased, BMI, total fat mass, and girth increased significantly. Percent total body fat correlated best with girth, suggesting that girth is a good indicator for measuring fat mass when DEXA and MRI are unavailable. The increase in body weight correlated well with an increase in food intake.

Glucose and Insulin

We have previously shown, when comparing obese cats with lean cats, that an increase in body weight by 1 kg leads to a decrease in insulin sensitivity by approximately 30%. However, a longitudinal study examining the insulin changes has not been performed to our knowledge. The lack of change in baseline glucose concentrations is likely due to the fact that obese cats can down-regulate hepatic glucose output (Kley et al 2009). However, AUC for glucose, although unchanged up to 30% body weight increase was significantly higher at 60% body weight increase in cats fed AOX, indicating glucose intolerance. A significant decrease was seen in insulin sensitivity with increasing body weight. Baseline insulin concentrations and total AUC increased significantly with increasing body weight, BMI and girth indicating insulin resistance even at a 10% body weight increase.

Lipid Analysis

Triglycerides increased significantly with increasing body weight, indicating a possible increase in VLDL, the major triglyceride carrier. NEFAs also increased; this suggests lower lipoprotein lipase and higher hormone sensitive lipase concentrations.

Enzyme Assays

We did not see any significant change in catalase, glutathione peroxidase, or SOD enzyme activity in RBCs or in heparin releasable SOD in plasma, not supporting our hypothesis that increased obesity would be associated with an increase in oxidative stress.

Cytokines

We saw no significant change in plasma IL-6, IL-1, and TNF-alpha with increasing body weight, not supporting a generalized inflammatory response to the increase in body weight.

Indirect Calorimetry

The RER increased significantly at 30 and 60% body weight increase, indicating a decrease in fat oxidation. Our results show a significant increase in heat production with increasing weight, indicating increased energy production, likely due to the increase in food intake.

Conclusions

An increase in obesity does not elicit a systemic inflammatory response in cats, despite the fact that it leads to insulin resistance even at a 10% body weight increase. These results may in part explain the fact that obese cats do no exhibit signs of the metabolic syndrome including atherosclerosis and hypertension.

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