

CHARACTERIZATION OF ADIPONECTIN IN THE CANINE

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CHARACTERIZATION OF ADIPONECTIN IN THE CANINE

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A Dissertation

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Doctor of Philosophy

Auburn, Alabama

August 4, 2007

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DISSERTATION ABSTRACT
CHARACTERIZATION OF ADIPONECTIN IN THE CANINE

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Doctor of Philosophy, August 4, 2007
(D.V.M., Auburn University College of Veterinary Medicine, 2001)
(B.S., Clemson University, 1997)

148 Typed Pages

Directed by Robert L. Judd

Obesity is the most common nutritional disorder observed in small animal medicine. Obesity in dogs can result in numerous complications, including traumatic and degenerative orthopedic diseases, cardiopulmonary compromise, heat and exercise intolerance, pancreatitis, glucose intolerance, and diabetes mellitus. Adiponectin is a 30 kD hormone produced exclusively by adipocytes that was identified independently by four different groups in 1995. Serum concentrations of adiponectin are high in the normal state, accounting for approximately 0.01-0.03% of total plasma proteins. However, circulating concentrations of this hormone are significantly reduced in obesity, insulin resistance and diabetes. Decreased adiponectin concentrations have been significantly correlated with the development of insulin resistance. Insulin resistance is often associated with obesity and may be one of the links between increased adiposity

and the development of type 2 diabetes. In dogs, the impact of compromised glucose tolerance and insulin resistance on health appears when dogs are even moderately overweight. The reduced capability to absorb glucose can hinder the ability of organs, tissues, and body systems to function properly, which can result in chronic health conditions. With increasing evidence that adiponectin may physiologically regulate energy metabolism and its importance in the relationship between obesity and the development of insulin resistance, adiponectin could potentially serve as a treatment target or marker for obesity, insulin resistance, and/or pre-diabetes. In order to determine the effects that insulin resistance has on adiponectin secretion and expression we have first characterized the secretion, circulating protein complex profile, and gene expression in normal dogs. Adiponectin is secreted into the serum as a >360 kD multimer, termed the HMW (high molecular weight) complex and as a 180 kD LMW (low molecular weight) complex. Under various reducing and denaturing conditions, adiponectin separates into cross-linked products whose molecular sizes are multiples of 30 kD. Additionally, gene expression of adiponectin is observed within visceral adipose tissue. We next studied adiponectin gene expression and protein secretion in dogs fed a high-fat diet in order to induce adiposity. High-fat fed dogs had a 2.6 fold increase in adiposity in the face of minimal weight gain (15.4%). High-fat fed dogs developed high/normal fasting insulin concentrations compared to controls at the peak of adiposity. Interestingly, adiponectin gene expression and secretion of total and high molecular weight adiponectin were unaffected at all timepoints during the course of this study.

ACKNOWLEDGEMENTS

I would like to express my deepest thanks to my major advisor, Dr. Robert L. Judd, for his guidance, understanding, and support as well as to other members of my committee, Dr. Stephen D. Lenz, Dr. Lauren G. Wolfe, and Dr. Robert J. Kemppainen. I would also like to thank Cindy Shaw, Jamie Butler, Tracey Land, Jacqueline Nobles, Emily Champion, Leah Ann Kuhnt, Misty Edmonson, Peter Christopherson, Colin Rogers, and Heather Gray, especially for their encouragement and friendship during this work. For their assistance in the laboratory, I would like to thank Dr. Tim Braden, Patricia DeInnocentes, Deepa Bedi, Eric Plaisance and Qiao Zhong. For their assistance in dog studies, I would like to thank Dr. Pat Rynders, Elliot Ramos Rivera, Andrea Muir, Melissa Ramos, Dr. Michael Tillson, Betty Files, and Dr. William Brawner. I would like to thank Dr. Kevin Huggins for being my outside reader. I would also like to express my deep appreciation and thanks to my parents, Lymon and Regena Brunson for their unconditional love and support during this process. Finally, great thanks go to all of the animals utilized for the production of this work. Without them, none of this would have been possible.

Style manual or journal used: American Journal of Physiology

Computer software used: Microsoft Word, Microsoft Excel, Slide Write Software, the Statistical Analysis System (SAS)

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CHAPTER I.

INTRODUCTION

Canine obesity is a common clinical entity and is believed to be caused primarily by long-term overeating and lack of exercise (28; 72). It has been suggested that at least 95% of cases of canine obesity are due to simple overeating (61). Complications of canine obesity include traumatic and degenerative orthopedic diseases, cardiopulmonary compromise, heat and exercise intolerance, glucose intolerance, diabetes mellitus, pancreatitis, and fatty infiltration of the liver (167). Studies have shown that total insulin secretion and fasting plasma insulin concentrations are highly significant linear functions of obesity (105; 125). Mattheeuws *et al.* demonstrated that dogs with approximately 40% body fat had normal glucose tolerance and insulin secretion; hyperinsulinemia was seen at higher degrees of obesity and only the group with the highest degree of obesity (approximately 75%) showed glucose intolerance. In another study by the same group, comparing the blood glucose and insulin concentrations of two groups of dogs during an intravenous glucose tolerance test, insulin secretion was lower in the obese diabetic group, despite a higher peak blood glucose concentration compared to the obese, non-diabetic group. This decompensation is similar to what has been described in cats and humans (72).

Canine diabetes is primarily observed in middle-aged and older dogs (121). Currently, there is no internationally accepted criteria for the classification of canine diabetes, so the terminology is inconsistent and challenging to interpret. Generally, there are three types of diabetes in the dog, divided into the categories of type 1 (insulin-dependent diabetes), type 2 (non-insulin-dependent diabetes), and diabetes secondary to other endocrinopathies (72). Type 1 diabetes in dogs is similar to type 1 diabetes in humans and is thought to be caused by autoimmune-mediated destruction of pancreatic beta cells. Dogs with this form of diabetes are prone to develop ketoacidosis and require insulin for survival. Canine type 2 diabetes is similar to that in humans. Mattheeuws *et al.* found that dogs with type 2 diabetes differed from dogs with type 1 diabetes only in their basal plasma initial insulin concentrations and that obese type 2 dogs had markedly higher initial insulin concentrations than non-obese type 2 dogs. The third type of diabetes occurs in connection with endocrinopathies; the most common being hyperadrenocorticism and acromegaly (72). Type 1 diabetes is the most common type of diabetes mellitus in the dog and, using established human criteria, at least 50% of diabetic dogs would be classified as type 1 with the remainder being classified as those that result from pancreatic destruction or chronic insulin resistance (144). It has been proposed that diabetes mellitus in adult dogs progresses through several stages beginning with impaired glucose tolerance, followed by a non-insulin-dependent stage resembling type 2 diabetes mellitus, and finally an insulin dependent disease comparable to type 1 diabetes mellitus (66).

In humans, substantial research has indicated that adipose tissue is not simply an energy storage organ, but is a secretory organ that produces a variety of proteins that

function to influence the metabolism of the body (2). These functions are mediated in part by secreted proteins, termed adipokines, that include leptin, resistin, adiponectin, tumor necrosis factor alpha (TNF- α), plasminogen activator inhibitor type 1 (PAI-1), adipisin, and IL-6, among others (53; 63). The recent discovery that the adipokine, leptin, circulates at increased concentrations in obese dogs, has peaked the interest of investigators in canine adipocyte biology and canine pathophysiology (84). Adiponectin is thought to link obesity and the development of insulin resistance in humans. Thus far, the relationship of adiponectin, obesity, and insulin resistance in the dog has not been studied.

Adiponectin was first identified in cultured adipocytes (rat, mouse, human) and human serum in 1995 by four independent laboratories. It circulates in the plasma in high concentrations ranging from 5-30 $\mu\text{g/ml}$ accounting for approximately 0.01-0.03% of total plasma proteins (2; 147). Adiponectin has been shown to increase beta-oxidation and decrease hepatic glucose output and is now widely thought of as an insulin-sensitizing hormone. One of the hallmark characteristics of type 2 diabetes is insulin resistance, defined as the reduced ability of insulin to promote glucose clearance in its target tissues and suppress hepatic glucose production. Significant decreases in circulating concentrations of adiponectin have been described in human type 2 diabetic patients and could account for a portion of the decreased insulin sensitivity observed in these patients. In humans, plasma adiponectin concentrations exceed those of any other hormone by a thousand times; adiponectin concentrations decrease with obesity and are positively associated with whole-body insulin sensitivity. Therefore, low adiponectin concentrations may contribute to the decrease in whole-body insulin sensitivity that

accompanies obesity (152) and it is necessary to understand the underlying mechanisms involved in the dysregulation of adiponectin in the obese/insulin resistant state.

To investigate whether insulin resistance plays a role in the secretion profile and gene expression of canine adiponectin, the protein was first characterized in 10 normal dogs by collecting serum samples through jugular venipuncture and a fat sample (falciform ligament) through a routine surgery. Normal circulating adiponectin concentrations were determined by radioimmunoassay and the stability of the protein was assessed over a time period of one year at different storage conditions (-80°C and -20°C) and under different collection procedures (as serum and plasma collected with EDTA or heparin anticoagulants). Adiponectin protein complex profiles were determined by SDS-PAGE and immunoblotting, and adiponectin gene expression was analyzed by reverse-transcription followed by polymerase chain reaction (RT-PCR). Adiponectin circulates at concentrations ranging from $0.85\ \mu\text{g/mL}$ to $1.5\ \mu\text{g/mL}$ in canine serum and exhibits a similar protein complex profile as humans, comprised of multiple cross-linked products that have molecular weights that are multiples of 30 kD under various reducing and denaturing conditions. The protein is stable in serum, EDTA plasma or heparinized plasma when stored for a period of one year at -80°C or -20°C . Additionally, we investigated adiponectin protein secretion and gene expression in control and high-fat fed dogs utilizing a human high molecular weight adiponectin ELISA, a canine adiponectin lincoplex assay, and real time PCR. Fourteen dogs were evaluated at 3 timepoints corresponding to baseline, increased adiposity, and decreased adiposity through measurement of body composition, intravenous glucose tolerance tests, dual energy x-ray absorptiometry, and surgical collection of adipose tissue. Body composition

measurements and DEXA scans revealed that high fat-fed dogs developed a significant degree of whole body adiposity (2.6 fold) at the peak of adiposity with minimal change in body weight (15.4%). Intravenous glucose tolerance test results identified that high-fat fed dogs at the peak of adiposity had high/normal fasting insulin concentrations, indicating minimal development of insulin resistance. Surprisingly, fasting glucose concentrations were unaffected at all timepoints during the course of the study. Serum and adipose tissue (from visceral and subcutaneous depots) obtained at these 3 timepoints were further analyzed for total and high molecular weight adiponectin concentrations (in serum) and adiponectin gene expression. Serum concentrations of total and high molecular weight adiponectin were unaffected by high-fat feeding during the course of the study. Furthermore, canine adiponectin gene expression in visceral and subcutaneous fat depots remained unchanged during the course of the experiment.

CHAPTER II.

REVIEW OF LITERATURE

Canine Obesity and Associated Disorders

The overweight dog is readily appreciated by the veterinary practitioner and several Western European surveys show that obesity is a common clinical entity. Excessive deposition of body fat has detrimental effects on the health and longevity of dogs (19). Obesity is a major concern because it predisposes dogs to numerous health problems including traumatic and degenerative orthopedic disorders (rupture of cruciate ligaments, hip dysplasia, and elbow problems), increased prevalence of cardiovascular disease (in the form of congestive heart failure), glucose intolerance, pancreatitis, diabetes mellitus, increased risk for the development of transitional cell carcinoma of the urinary bladder, anesthetic complications, decreased heat tolerance and stamina, dyspnea, dystocia, dermatologic problems, and decreases in immune function. Obesity can also be a clinical sign that accompanies various endocrinopathies including hyperadrenocorticism, hypothyroidism, hyperinsulinemia, hypogonadism, and diabetes mellitus (19; 167). It is unclear whether the relationship of canine endocrinopathies to obesity reflects overeating, under-exercising, or real changes in metabolic efficiency associated with the endocrinopathy.

Hypothyroidism is believed to cause a reduction in resting metabolic rate; hyperadrenocorticism and hyperinsulinemia are associated with polyphagia; hyperadrenocorticism results in increased fat storage; and hypogonadism is associated with reduced energy expenditure (167). In dogs, obesity causes insulin resistance, which leads to hyperinsulinemia and impaired glucose tolerance (142). These effects are particularly pronounced when obesity is induced by feeding a diet high in saturated fat (155). A complete list of the disorders associated with or exacerbated by obesity is listed in Table 1.

<p>Metabolic Alterations Hyperlipidemia Insulin Resistance Glucose Intolerance Anesthetic Complications</p> <p>Endocrinopathies Hyperadrenocorticism Hypothyroidism Diabetes Mellitus Insulinomas Pituitary Chromophobe Adenoma Hypopituitarism Hypothalamic Lesions</p> <p>Functional Alterations Joint Stress/Musculoskeletal Pain Dyspnea Hypertension Dystocia Exercise Intolerance Heat Intolerance Decreased Immune Function</p> <p>Other Diseases Degenerative and Orthopedic Diseases Cardiovascular Disease Transitional Cell Carcinoma (bladder)</p>

Table 1: Diseases associated with or exacerbated by obesity. Adapted from Burkholder WJ, Toll PW. Obesity. *Small Animal Clinical Nutrition IV*. 1997;1-43.

Canine Obesity

Overconsumption of calories resulting in excess body fat is believed to be the most prevalent form of malnutrition in pets of westernized societies. Obesity is a pathological condition characterized by the accumulation of adipose tissue in excess of that required for optimal body function. Excess adipose tissue is formed when energy intake chronically exceeds energy expenditure and this imbalance is typically attributed to overeating, under-exercising, or a combination of both factors. It has been suggested that at least 95% of canine obesity is due to simple overeating (167). Results from a large survey done in the United States indicated that 25% of 23,000 dogs from 60 private veterinary practices were overweight or obese (19). Dogs that have an accumulation of body fat that puts them 20% or more over ideal body weight are considered obese. These dogs often have fat deposits over the thorax, spine, and the base of the tail, as well as the neck and limbs (107). Canine obesity can be classified as “uncomplicated” and “complicated” which are utilized to distinguish between adiposity that is due to overeating/under-exercise and that resulting from other causes. Factors that contribute to uncomplicated obesity include gender, sexual integrity, age, and breed. Females are more likely to be overweight than males, while neutering predisposes both sexes to obesity (28). Energy intake does not appear to be influenced by neutering, suggesting that changes in energy expenditure, in terms of exercise, or efficiency of energy utilization are responsible for the development of obesity in neutered dogs (61). The prevalence of obesity in dogs increases after two years of age and reaches a maximum around six to eight years of age and subsequently plateaus until 12 years of age at which time, the prevalence tends to decrease markedly (19). Researchers have found that obesity in

people has a large propensity for being heritable, and clearly, some dog breeds are more likely to be overweight compared to others. Labrador Retrievers, Cairn Terriers, Cocker Spaniels, Dachshunds, Shetland Sheepdogs, Bassett Hounds, Cavalier King Charles Spaniels, and Beagles have a greater prevalence of obesity than other breeds (38; 122). The predisposition of certain breeds to obesity suggests a familial etiology, but no definitive genetic factor has been identified in dogs. The identification of genes and gene products, such as leptin and neuropeptide Y that regulate energy balance in humans and rodents has re-awakened interest in genetic causes of obesity. It has been shown that the leptin gene exists in the dog, suggesting that other homologous genes may exist and defects in these genes or the receptors with which their products interact may result in ineffective regulation of energy intake and metabolism (84; 85). “Complicated” obesity is most often associated with endocrinological diseases. Generalized obesity is sometimes the only obvious clinical sign early in the course of hyperadrenocorticism. Paradoxically, most dogs with hyperadrenocorticism are not obese in the strictest sense, but rather the impression of adiposity is given by abdominal enlargement and redistribution of pre-existing fat. Hypothyroidism is a relatively common canine disease in which obesity occurs frequently. Overweight dogs tend to develop diabetes mellitus (28). Determining whether a dog is overweight would not seem to be a very challenging task, however, in the clinical setting, the subjectivity inherent in such a determination makes irrefutable, objective measurement challenging. The subjectivity results from variation in body conformation across breeds, variation in frame size within breeds, as well as the veterinarian’s and/or owner’s bias for what is an ideal body weight and conformation of

the pet. A variety of methods are available for body composition measurement in research settings and clinical settings (Table 2).

Body Composition Measurement Techniques	
<u>Research</u> Magnetic Resonance Imaging (MRI) Computed Tomography (CT) Neutron Activation Hydrodensitometry Deuterium Oxide Dilution Total Body Potassium Ultrasound Bioelectrical Impedance Dual Energy X-ray Absorptiometry	<u>Clinical</u> Relative Body Weight (RBW) Body Condition Score (BCS) Morphometric Analysis

Table 2: Common research and clinical methods utilized to measure body composition in dogs. Adapted from Burkholder WJ, Toll PW. Obesity. *Small Animal Clinical Nutrition IV*. 1997;1-43.

The methods described above all have advantages and disadvantages. Metabolic differences between subcutaneous and visceral adipocytes may partly explain the association between adipose tissue distribution and disease, emphasizing the need for methods by which adipose tissue distribution in the body can be studied.

Deuterium Oxide Dilution

The deuterium oxide dilution (DOD) method is accepted as the most reliable method for body fat assessment. Deuterium oxide, a stable isotope, distributes water rapidly to the whole body, and then it is possible to calculate the body water volume from the dilution factor. However, deuterium oxide does not distribute to fat; thus the fat volume can be calculated by subtraction from whole body volume (20; 82). The DOD

method is not practical for clinical use because it takes considerable time and the laboratories that can measure the deuterium oxide are limited (82).

Computed Tomography

A method based on taking transaxial images at several well-defined locations by computed tomography (CT) and measuring the area of adipose tissue in each image has been utilized to calculate the volume of adipose tissue in these areas. This method is of interest because it has been demonstrated that the location of adipose tissue in the body is important for the health hazards associated with obesity. Abdominal obesity (defined as an increased waist-hip circumference ratio) has been shown to be associated with cardiovascular disease, stroke, non-insulin dependent diabetes mellitus as well as endometrial and ovarian carcinomas in people. However, because of the radiation required for a CT scan, the number of measurements that can be performed on one individual is limited, making this method unsuitable for studies requiring repeated measurements of the same subject.

Magnetic Resonance Imaging

Body adipose tissue can also be studied by the magnetic resonance imaging (MRI) technique, in which images are created by a combination of electromagnetic radiation and a magnetic field. The amount and distribution of adipose tissue can be assessed from the images in the same way as in the CT method, but no radiation hazards are involved, therefore allowing examinations to be carried out repeatedly in the same subject. A drawback with the MRI technique is the high cost of the equipment required (148).

Dual Energy X-ray Absorptiometry

Dual energy x-ray absorptiometry (DEXA) is unique in that it permits accurate determination of body composition (including bone mineral density and fat and lean mass) in live animals by use of low-dose radiation and without subject discomfort. DEXA uses an x-ray tube and filters to produce a pencil-beam with 2 energies that are attenuated differently by each tissue type. Computerized algorithms quantify the various components of body composition: bone mineral content corresponds to the 60% of hydrated bone tissue that is mineral and correlates well with bone ash; soft tissue consists of all tissue not included in the category of bone and is composed of fat and lean mass. Fat tissue (essentially water-free) may be separated from lean mass, which includes all hydrated tissues other than bone mineral. The extremely low radiation exposure permits serial analyses of treatments or disease states during extended periods with minimal risk to the patient. Presently, few DEXA instruments are available in veterinary research and clinical centers, in large part because of initial capital costs, lack of experienced personnel, and lack of defined clinical or research applications (108).

Bioelectrical Impedance

A weak electric current is passed through the body and resistance is measured. Modern BIA methods are all based on a similar principle: resistance to an applied alternating electrical current is a function of tissue composition. The various tissues the current travels through affect the final measurement resulting in estimates of fat, fat-free mass, and intra- and extra-cellular water.

Neutron Activation

Neutron activation, or neutron inelastic scattering, is a method of measuring body composition that relies on a correlation between a carbon-oxygen ratio in the body and the percent body fat with corrections made for a level of patient hydration in the lean tissue. The patient is irradiated with neutrons having sufficient energy to inelastically scatter off of carbon and oxygen. The resulting gamma rays are detected and counted. The ratio of the detected gamma rays and hydration are then related to a proportion of fat in the animal body. This technique provides accuracy approaching that achieved with hydrodensitometry with only small radiation doses between 5 and 20 millirem. Where relevant, gamma rays from different regions of the animal body may be detected and separately accounted. This enables the determination of the proportion of fat in those regions, allowing for the measurement of regional body fat and fat distribution.

Hydrodensitometry

The calculation of body composition from measures of body density is the classic method with which most persons are familiar. Traditionally it involves submerging the subject in a water tank and measuring the subject's weight while under water. The two-component model assumes that body weight consists of fat and fat-free mass. The density of fat is well established in both humans and animals and is approximately 0.9 g/cm^3 . Only minimal variation is recognized within and between species in the density of fat. This is because fat, or specifically lipid, is almost entirely triglycerides. On the other hand, fat-free mass is a heterogeneous compartment consisting of at least four major components including water, protein, glycogen, and minerals. The density of these components varies from a low of 0.994 g/cm^3 for water to a high of 3.04 g/cm^3 for

minerals. Siri and other workers later refined these densities such that fat is now assumed to have a density of 0.9 g/cm^3 and fat-free mass a density of 1.1 g/cm^3 at body temperature. Assuming these two known and constant density's and given the subjects measured density by underwater weighing, one can then compute the percentage of body weight as fat. The extent that these proportions change in any individual subject will introduce corresponding errors in the assumed density of fat-free mass. A number of studies suggest that the density of fat-free mass is relatively stable across age and sex groups, although some variation is recognized at the extremes of age and in patients who have underlying medical and surgical conditions. The hydrodensitometry or underwater weighing method served as a reference technique for at least four decades against which other methods were compared. The importance of the underwater weighing method is that measurement systems are relatively inexpensive to construct, simple to operate, and the procedure is safe for patients varying widely in age and body weight. The method's disadvantages for both human and animals are that some subjects have concerns about water submersion and thus either decline or refuse to cooperate. Underwater weighing is also a technique that by necessity is stationary and cannot be moved around for field applications. A reasonable amount of technician skill is required to perform the method that under optimal conditions has a small technical error of about 0.001 g/cm^3 . The residual lung volume measurement can also be difficult to obtain from both human and animal patients and adds to the measurement error, thus adding to the method's total error.

Total Body Potassium

Estimation of total body potassium (TBK) is recognized as a classical method of

quantifying total body fat. Potassium in nature occurs as three isotopes, ^{39}K (93.1%), ^{40}K (0.0118%), and ^{41}K (6.9%). The most abundant forms, ^{39}K and ^{41}K , are non-radioactive while the naturally-occurring radioactive potassium isotope ^{40}K ($t_{1/2}=1.3 \times 10^9$ years) gives off a 1.46 MeV gamma-ray that can be counted using detectors such as crystalline sodium iodide. The proportion of total potassium found in human tissues as ^{40}K is constant at 0.0118% of total potassium. Thus, by measuring ^{40}K one can compute total-body potassium (TBK). In turn, potassium is distributed almost entirely within the intracellular compartment of fat-free mass. As the ratio of total-body potassium to fat-free mass is relatively stable in adult humans, one can compute fat-free mass and total-body fat if TBK is known. Forbes and his colleagues were the first to report in 1961 the measurement of ^{40}K and thus TBK using a whole-body counter. From measured TBK Forbes and his colleagues proposed the use of the relatively stable TBK to fat-free mass ratio as a means of estimating in vivo fat-free mass and total-body fat. The whole body ^{40}K counting method became the reference approach for evaluating total-body fat for several decades. While whole body counters are very costly instruments to install, their operational expenses are relatively small. Systems are simple to operate and there are no recognized health risks. As the regional and whole body counters are extremely heavy, they cannot be used outside of specialized research laboratories dedicated to the study of metabolic diseases and human body composition. Whole-body counters are not widely available, the measurements are sometimes time consuming, and with some systems the procedure may be difficult for subjects.

Ultrasound

Many methods of ultrasonography have been utilized to quantify fat mass in animals and humans. Ultrasound measurements of visceral and subcutaneous fat are defined by determining the distance between the skin and external face of the rectus abdominis muscle for subcutaneous fat, and the distance between the internal face of the same muscle and the anterior wall of the aorta for visceral fat (143). Additionally, obesity can be estimated by two dimensional ultrasonograms of back fat measured in the transverse planes on the top of the spinous process of the 6th and 7th lumbar vertebrae and the 1st sacral vertebra (127). Ultrasound has the advantages of being low cost and safe (patients are not exposed to ionizing radiation), However, the variability and lack of correlation to measurements obtained by more standard methods (such as CT or MRI) make ultrasound a slightly less desirable method for quantifying fat mass.

Insulin Resistance

Insulin is a major metabolic hormone produced by the β -cells of the pancreas. It exerts its biological effects through interaction with specific cell surface receptors. The insulin receptor is a disulfide-linked heterotetrameric glycoprotein composed of two extracellular α -subunits containing insulin-binding sites, and two transmembrane spanning β -subunits possessing tyrosine kinase activity in the catalytic domains. Binding of insulin to its receptor triggers a series of phosphorylation events crucial to insulin action. Following insulin binding, the receptor undergoes autophosphorylation on tyrosine residues of the intracellular portion of the β -subunit, which increases the receptor's intrinsic tyrosine kinase activity. Activation of the insulin receptor results in phosphorylation, and subsequent activation of insulin receptor substrate-1 and -2 (IRS-1

and IRS-2). Phosphorylation of IRS-1 and IRS-2 generates secondary signals including phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK), leading to an increase in glucose transport, glycogen synthesis, fatty acid synthesis and decreased lipolysis (90).

Following a meal, insulin is released, resulting in cellular glucose uptake and conversion to glycogen or triglycerides (60). Insulin inhibits gluconeogenesis and glycogenolysis, and promotes glycogen synthesis in the liver. It also reduces free fatty acid release from adipose tissue, partially through inhibition of hormone sensitive lipase. Also in adipose tissue, insulin decreases lipolysis and increases the rate of synthesis of triglycerides from non-esterified fatty acids (NEFA) (177). During fasting, circulating insulin levels decrease, glycogen is mobilized and glucose use is primarily limited to tissues of the central nervous system for energy. Other tissues begin to oxidize free fatty acids released from adipose tissue for energy.

Insulin resistance appears to be the major common finding in individuals with obesity, glucose intolerance, or type 2 diabetes, high blood pressure, and dyslipidemia. It has been suggested that insulin resistance may be the initial factor triggering a metabolic cascade which is also influenced by genetic and environmental factors (62). Studies examining the relationship between insulin resistance and central versus subcutaneous fat accumulation have shown that there is a higher incidence of insulin resistance in individuals with central obesity than those with subcutaneous obesity (101). Insulin resistance is the consequence of a decrease in the number of insulin receptor sites and a post-receptor defect and is characterized by decreased responsiveness to insulin at its target tissues, including skeletal muscle, liver, and adipose tissue (55). Insulin resistance

decreases the ability of insulin to suppress hepatic glucose production as well as promote glucose metabolism and clearance (90). As a result, the β -cells of the pancreas produce increased amounts of insulin to maintain glucose homeostasis. This results in a condition known as hyperinsulinemia. Eventually the β -cells can no longer produce enough insulin to compensate for the increase in circulating glucose concentrations, and the result is type 2 diabetes. Insulin resistance combined with β -cell failure leads to the decompensated hyperglycemic diabetic state (90). Reduced insulin-mediated glucose uptake has been confirmed in overweight dogs by euglycemic hyperinsulinemic clamp studies (55). In a recent canine longevity study, diet-restricted dogs had lower basal circulating glucose and insulin concentrations and these parameters were shown to be positively related to survival time until death and low hazard of dying from or needing treatment for chronic disease. Optimum glucose tolerance and insulin response appear to be integrally involved in the health and longevity of dogs. In this study, body fat mass and insulin sensitivity were highly correlated, suggesting that long term control of food intake to minimize circulating glucose and enhance insulin response will improve the quality and increase the quantity of the dog's life (107).

Diabetes Mellitus

Canine diabetes is mostly found in middle aged and older dogs (121). The etiology of diabetes mellitus in dogs is probably multifactorial, with factors including obesity, diet, exposure to toxic chemicals or drugs that cause insulin resistance, immune-mediated destruction of islet cells, and destruction of islet cells secondary to pancreatitis all playing a possible role in the disease. While genetic factors probably influence susceptibility, specific genes and inheritance patterns have not been identified in most

dog breeds. Breeds that are reported to have a higher risk for developing diabetes mellitus include Keeshond, Alaskan Malamute, Puli, Cairn Terrier, Fox Terrier, Manchester Terrier, Miniature Pinscher, Toy and Miniature Poodles, Finnish Spitz, Schipperke, and Miniature Schnauzer. Pekingese, German Shepherd, Collie, and Boxers are reportedly at decreased risk (66). A 2003 study by Guptill *et al.* indicated that the number of patients presented to veterinary teaching hospitals for diabetes mellitus has increased since 1970 (from 19 cases per 10,000 admissions per year in 1970 to 64 cases per 10,000 admissions in 1999) despite the fact that diagnostic methods and criteria have not changed over this time period. This suggests that the increased hospital prevalence is not a result of changes in clinical practice, but rather reflects a true increase in the incidence of diabetes mellitus in the dog population. Obesity was identified as the most important factor influencing glucose tolerance and insulin response in both healthy and diabetic dogs (125).

At present there is no internationally accepted criteria for the classification of canine diabetes, so canine diabetes terminology is rather inconsistent and challenging to interpret. Generally, there are three main types of diabetes in the dog that are divided into the categories of type 1 (insulin-dependent diabetes), type 2 (non-insulin dependent diabetes), and diabetes secondary to other endocrinopathies (72). Type 1 diabetes in dogs is similar to type 1 diabetes in humans and is thought to be caused by autoimmune mediated destruction of beta cells. Dogs with this form of diabetes are prone to develop ketoacidosis and need insulin for survival. Type 2 diabetes is somewhat similar to that of type 2 in humans. Mattheeuws *et al.* found that type 2 dogs differed from type 1 dogs only in their basal plasma initial insulin concentrations and that obese type 2 dogs had markedly higher initial insulin concentrations than nonobese type 2 dogs (125). The third

type of diabetes occurs in connection with endocrinopathies; the most common being hyperadrenocorticism and acromegaly (72). Type 1 diabetes is the most common form of diabetes mellitus in the dog and, using established human criteria, at least 50% of diabetic dogs would be classified as type 1 with the remainder being classified as those that result from pancreatic destruction or chronic insulin resistance (142). Diabetes mellitus affects the domestic canine in much the same way that it affects man, with the classical array of clinical and biochemical manifestations appearing in both species. Though the prevalence of type 2 diabetes in dogs is thought to be extremely low, middle aged and older dogs exhibit insulin responses that resemble those found in human adult onset (or type 2) diabetes mellitus, which suggests it may occur at a much higher rate than previously believed. It has been proposed that diabetes mellitus in adult dogs progresses through several stages beginning with impaired glucose tolerance, followed by non-insulin dependent stages resembling type 2 diabetes mellitus in human beings, and finally an insulin dependent disease comparable to type 1 insulin dependent diabetes mellitus of human beings (66). In support of this proposed mechanism Chin *et al.* have also shown that during intravenous glucose tolerance tests, the initial stage of insulin secretion in chemically diabetic dogs is greatly diminished even though the prevailing glucose load demands an increased supply of the hormone; this is followed by a temporally inappropriate enhancement of insulin release which appears to result in a transient hyperinsulinemia. This response resembles that observed in man, and is particularly informative in revealing the presence of pre-clinical diabetes, where pancreatic damage and/or atrophy is not severe. Findings relative to this stage are particularly important because it is likely that the diabetic animal with subclinical impairment of glucose

tolerance is not detected and it is precisely this stage which is potentially amenable to the institution of corrective measures (25).

Canine Atherosclerosis

Obesity, diabetes mellitus, gender, and age are considered to be risk factors for the development of atherosclerosis in dogs (89). Naturally occurring and experimental atherosclerosis in dogs is uncommon and almost always occurs in association with hypothyroidism. (65; 88). It has been demonstrated that when dogs develop atherosclerosis, the disease closely resembles that of the human disease subsequent to the development of hyperlipoproteinemia (118). Despite similarities, the dog differs from humans in that they are substantially resistant to the development of atherosclerosis and hypercholesterolemia (almost always occurring in association with hypothyroidism) and canine serum is rich in HDL compared to human serum rich in low density lipoproteins (LDLs) and very low density lipoproteins (VLDLs) (119).

Adipose Tissue

Adipose tissue is the body's largest energy reservoir. The primary role of adipocytes is to store triacylglycerol during periods of caloric excess and to mobilize this reserve when expenditure exceeds intake. Mature adipocytes are uniquely equipped to perform these functions, possessing the full complement of enzymes and regulatory proteins needed to carry out both lipolysis and de novo lipogenesis (53). This classical view of adipocyte function was re-evaluated with the discovery of the cytokine-like factor, leptin, which is secreted principally from adipocytes, and acts centrally upon the hypothalamus and peripherally upon other organs (176). We now know that adipose tissue is much more complex than previously thought, and that it operates as an endocrine

organ that releases hormones in response to specific extracellular stimuli or changes in metabolic status. At present, adipocytes are known to produce well over 20 proteins that are involved in inflammatory, metabolic, and endocrine functions. These secreted proteins perform diverse functions, but seem to share some structural properties of cytokines. They have therefore been referred to collectively as “adipocytokines”. Most of these factors secreted from adipose tissue act in an autocrine/paracrine manner to regulate adipocyte metabolism; upon secretion into the bloodstream, they act as endocrine signals at multiple distant sites to regulate energy homeostasis (95).

White adipose tissue is a heterogeneous organ, and this is the case both in terms of differences between individual depots and in the range of cells that are present within the tissue. White adipose tissue is composed of several cell types, including mature white adipocytes and a stromal-vascular fraction that includes fibroblasts, endothelial cells, and macrophages. Adipocytes from visceral or subcutaneous depots largely differ concerning their metabolic characteristics in the control of lipolysis and the sensitivity of insulin. Visceral adipose tissue has consistently been shown to be associated with multiple abnormalities in insulin signaling, glucose intolerance, hypertension, and dyslipidemia (128). It has been shown that surgical removal of visceral adipose tissue improves insulin’s effect on hepatic glucose production in animal models of obesity (4). Subcutaneous adipose tissue contains adipocytes that are more lipolytically active, exhibiting elevated basal and β -adrenergically stimulated lipolysis (87).

Beside the energy-storing white adipose tissue, mammals possess a specialized type of fat, brown adipose tissue that has separate physiological functions. Brown adipose tissue is characterized by its thermogenic functions because it has the ability to dissipate

energy and to provide heat. Therefore, brown adipose tissue plays a major role in the regulation of body temperature, especially in small animals (such as mice or rats) or in infants (160). Brown adipocytes have also been shown to have endocrine function as they are able to express and secrete leptin into their environment (22).

The importance of adipose tissue as an endocrine organ and its role in the maintenance of energy homeostasis has become more and more apparent with increasing incidences of obesity, or excess adipose tissue. During the development of obesity many of the secreted factors from adipose tissue become dysregulated.

Adipose-derived proteins

Adipose-derived proteins, or adipokines, are circulating factors released from various cell types that form adipose tissue. Adipokines play important regulatory roles in a variety of complex processes, including fat metabolism, feeding behavior, hemostasis, vascular tone, energy balance, and insulin sensitivity (145). Circulating adipokines such as adiponectin, resistin and leptin are hormones released from mature adipocytes (43), while other adipokines such as IL-1 and IL-8 are released from nonfat cells such as stromovascular cells (43). TNF- α , PAI-1 and IL-6 are examples of adipokines released from both adipocytes as well as non-fat cells (43). Many of these adipokines send signals to the brain or peripheral organs indicating the mass of adipose tissue in the body as well as the state of energy homeostasis of the animal.

Although adipose tissue was identified as a major site of metabolism for sex steroids as early as 1987, it was not until the identification of leptin in 1994 that solidified the existence and importance of adipose-derived endocrine factors. An elegant series of parabiosis studies using *ob/ob* and *db/db* mice suggested that there was a factor secreted

from the periphery that had the ability to communicate with the brain and central nervous system regarding the status of energy stores in the body (31). This factor was later named leptin. The leptin gene was identified in 1994 and its receptors in 1995 and 1996 (24; 109; 154; 176). Leptin is derived from the Greek word “leptos” meaning thin. It is a 16 kDa hormone containing 167 amino acids with structural homology to a number of cytokines (93). Leptin is secreted from mature adipocytes in direct proportion to adipose tissue mass as well as to nutritional status; leptin secretion increases following the ingestion of food intake and has been shown to inhibit food intake as well as increase energy expenditure. Leptin levels begin to decrease proportionally as time increases following the ingestion of a meal, thus removing the inhibition of food intake, and decreasing energy expenditure (93). Leptin is increased by a variety of circulating factors including insulin, glucocorticoids, TNF- α , and estrogen. It is decreased by factors such as free fatty acids, androgens, growth hormone and peroxisome proliferators-activated receptor- γ agonists (PPAR- γ) (120).

It is now recognized that adipokines have many different functions in the body. Table 3 describes many of the known adipokines and their endocrine functions. These functions include sending signals regarding the nutritional status of the animal (leptin, adiponutrin), immune-related functions (interleukin-6), and regulation of blood flow (angiotensinogen).

ADIPOCYTE-DERIVED PROTEINS	TYPE OF PROTEIN/ ENDOCRINE FUNCTIONS
Leptin	Central satiety signal, hematopoiesis, reproduction, immune responses.
Tumor necrosis factor- α (TNF- α)	Cytokine, development of insulin resistance
Interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1)	Immune-related proteins
Plasminogen activator inhibitor-1 (PAI-1)	Proteins involved in fibrinolytic system, causes vascular thrombosis
Adipsin (complement factor D), Complement factor B, Acylation stimulating protein (ASP)	Complement and complement-related proteins
Lipoprotein lipase (LPL), Cholesterol ester transfer protein (CEPT), Apolipoprotein E, Non-esterified fatty acids (NEFAs)	Lipids and proteins for lipid metabolism or transport
Cytochrome P450-dependent aromatase, 17 β -hydroxysteroid dehydrogenase (HSD), 11 β hydroxysteroid dehydrogenaseHSD1	Enzymes involved in steroid metabolism
Angiotensinogen	Proteins of the renin angiotensin system, Regulation of blood pressure
Resistin	Development of insulin resistance and decreased glucose tolerance
Acylation –stimulating protein (ASP)	Promotes triglyceride synthesis
Adiponutrin	Energy homeostasis
Transforming growth factor- β (TGF- β)	Growth factor

Table 3. Adipocyte-derived proteins and their functions. Modified from Kershaw and Flier, *The Journal of Endocrinology and Metabolism* 2004. 89(6):2548-2556.

Adiponectin

Discovery

Adiponectin is a 30 kD protein that is exclusively secreted by adipocytes. It was originally discovered by four independent laboratories in the mid 1990's utilizing different experimental approaches (76; 115; 131; 147). Because of this, adiponectin is also known as Acrp30 (Adipocyte Complement Related Protein of 30kD), AdipoQ, apM1, and GBP28 (Gelatin-Binding Protein of 28 kD). In 1995, Scherer *et al.* identified a protein using subtractive cDNA screening to identify mRNAs induced during differentiation of 3T3-L1 adipocytes. Northern blot analysis of one specific clone showed a 100-fold increase in induction during differentiation; the resulting full-length cDNA was isolated and sequenced and the protein encoded by this cDNA was discovered to be novel. It was subsequently described as having sequence and structural homology to complement factor C1q, and therefore was named adipocyte-derived complement-related protein of 30kDa, or Acrp 30 (147). Scherer *et al.* were the first to suggest that Acrp30 secretion is acutely regulated by insulin, as well as the fact that it is a very abundant plasma protein, accounting for approximately 0.03% of total plasma proteins (147). Plasma adiponectin concentrations in humans range from 5-30 µg/mL, which are about 1000 fold higher than the concentrations of most other hormones, including leptin and insulin (68).

In 1996, Hu *et al.* used mRNA differential display to isolate a novel adipose cDNA they named adipoQ (76). The expression of adipoQ was specific to adipose tissue in both mouse and rat, and encoded a protein consisting of 247 amino acids with significant homology to complement factor C1q. The encoded protein contained a

collagenous structure in its N-terminus as well as a globular domain in its C-terminus.

AdipoQ was found to be highly regulated during the differentiation of adipocytes, and Hu *et al.* were the first to describe a significant down-regulation of adipoQ mRNA in fat tissues collected from obese humans and mice (76).

Maeda *et al.* isolated a novel adipose-specific gene, Adipose Most Abundant Gene Transcript (apM1), from human adipose tissue (115). The transcript of apM1 is the most abundant in all mRNA from adipose tissue. Northern blotting of RNAs from several different human tissues including skeletal muscle, small intestine, placenta, uterus, ovary, kidney, liver, lung, brain, heart and bladder revealed that apM1 was specifically expressed in adipose tissue. Further studies revealed that apM1 encodes the adiponectin protein.

In 1996, Nakano *et al.* used gel chromatography to identify a novel protein from human plasma they named gelatin binding protein of 28kDa or GBP28 (131). GBP28 was identified by its affinity to gelatin-Cellulofine. The protein purified from the column was described as having a molecular mass of 28kDa under reducing conditions, and 68kDa under non-reducing conditions. Further analysis revealed that the cDNA clone apM1 previously discovered by Maeda *et al.* encoded a protein containing all the sequences of GBP28 and it was concluded that apM1 encoded GBP28 (131). The amino acid sequence for GBP28 showed 82.7% homology to Acrp30 which is thought to be the murine equivalent of GBP28 (131).

Protein structure

The basic structural subunit of human adiponectin is a monomer which has a molecular weight of approximately 30 kD and consists of ~244 amino acids (147). It has

an N-terminal signal sequence, a non-homologous domain, a unique amino-terminal collagenous domain followed by 22 Gly-X-Pro or Gly-X-X repeats; suggesting that adiponectin has a straight chain collagen stalk (147). Adiponectin possesses a globular domain in the carboxy-terminus that bears structural homology to a number of other proteins, including the globular domains of type VIII and X collagens, and the subunits of complement factor C1q and TNF- α (147). Adiponectin's three dimensional structure is strikingly similar to that of TNF- α (165). At the amino acid level, adiponectin exhibits a substantial degree of homology among different species. For example, porcine adiponectin is 79-83% similar to the dog, human, cow, and mouse protein (86).

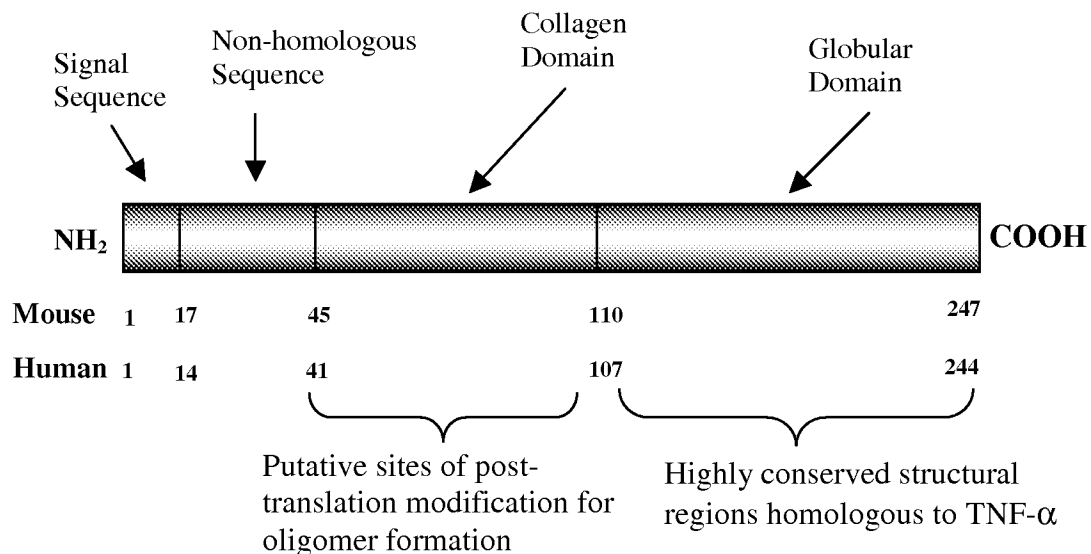


Figure 1: Schematic representation of murine and human adiponectin. Adapted from Ukkola O. and Santaniemi M. *Journal of Molecular Medicine*. 2002. 80:696-702, and Berg A. *et al. Trends in Endocrinology and Metabolism* 2002. 13:84089.

Adiponectin is secreted from adipocytes and post-translationally modified into many different isoforms (165). Two-dimensional gel electrophoresis of recombinant

adiponectin produced by *E. coli* revealed only one isoform of adiponectin suggesting that multiple isoforms of adiponectin are the result of post-translational processing in mammalian adipocytes (165). Adiponectin secreted from 3T3-L1 adipocytes is post-translationally modified into eight different isoforms. Six of these are glycosylated and subsequently hydroxylated at four lysine residues (68, 71, 80, and 104) located in the collagenous domain, while two are unglycosylated (164). This may account for or contribute to the heterogeneity of the protein. Wang *et al.* revealed that the glycosylation was neither *N*-linked nor *O*-linked, however the glycosylation occurred on 4 lysine residues in the collagen domain of the protein (165). Replacement of the lysine residues with arginine residues resulted in decreased action of insulin to suppress hepatic glucose production indicating that the glycosylated lysine residues are required for the biological activity of adiponectin (165). In later studies, Wang *et al.* purified adiponectin from fetal bovine serum and demonstrated that four proline residues in the collagenous domain are hydroxylated (Pro39, 42, 48 and 86), and a total of five lysine residues in the collagenous domain are hydroxylated and subsequently glycosylated (Lys28, 60, 63, 72 and 96) (164). Notably, the four hydroxylated and glycosylated bovine lysines (60, 63, 72, and 96) are equivalent to lysines 68, 71, 80, and 104 of murine adiponectin from 3T3-L1 adipocytes (164).

Adiponectin circulates in the serum as structures called protein complexes.

Adiponectin is secreted and circulates in serum as four protein complexes; a trimer (~90 kD), an albumin binding trimer (~100 kD), a low molecular weight (LMW) protein complex (~180 kD), and a larger, high molecular weight (HMW) protein complex (≥ 360 kD) (37; 137; 147). Figure 2 describes the formation of these complexes. Monomers are

single 30kDa proteins which can form trimers through associations in their globular domains (9). Trimers associate with other trimers through interactions in their collagen domains to form higher molecular weight structures (9). Adiponectin has been identified in human serum in three higher order structures: a trimer of 90 kD (in which two of three monomers are covalently linked by a disulfide bond between cysteine residues at position 22), a dimer of trimers (known as the low molecular weight (LMW) complex, approximately 180 kDa) or higher order structures which are larger complexes of 12-18 subunits (4-6 trimers, approximately 360-540 kDa) (136; 157). These higher order complexes are known as high molecular weight (HMW) complexes. The assembly of LMW and HMW forms of adiponectin depends upon the formation of Cys²²-mediated disulfide bonds. The LMW and HMW complexes form inside the adipocyte and are secreted into the circulation where they may undergo further proteolytic cleavage. For example, the globular domain of adiponectin has been identified in human plasma at physiologically significant levels and has been identified as having biological activity (23; 77).

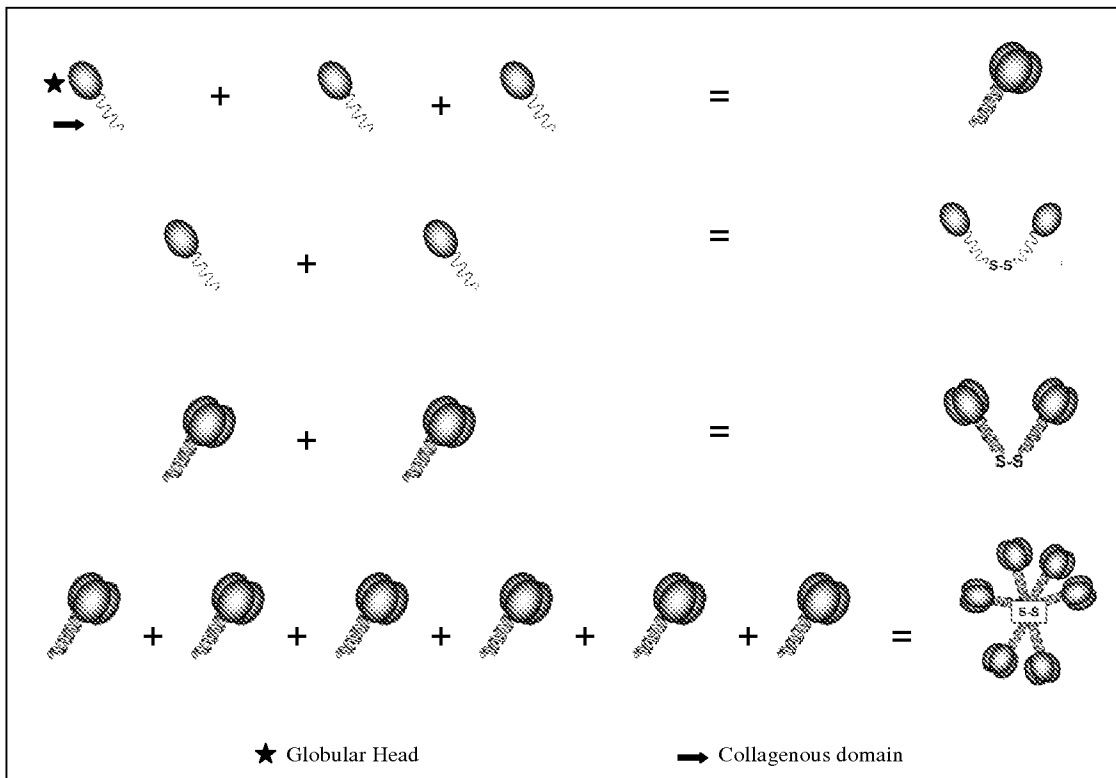


Figure 2: Model for assembly of adiponectin complexes. Three monomers form a trimer through associations between their globular domains. Four to six trimers associate noncovalently through their collagenous domains to form high-molecular weight oligomers, which circulate in the plasma. Adapted from Chandran *et al. Diabetes Care.* 2003.26(8):2442-2450. The albumin-binding trimer is not shown.

In addition to albumin, eight serum proteins have been identified which may directly or indirectly bind to adiponectin (Table 4). Interestingly, most of these proteins are extracellular matrix proteins involved in regulating tissue remodeling, angiogenesis, and inflammation. Adiponectin interacts directly and specifically with thrombospondin-1 (TSP-1) and alpha 2 macroglobulin (alpha2M) and it has been suggested that these proteins might serve as the physiological binding partners of adiponectin and regulate its bioavailability and biological activities (166).

Protein	Molecular Weight (kD)
Alpha2M precursor	163
H factor 1 (complement)	139
Platelet factor 4	11
Fibronectin precursor	262
Integrin	133
TSP-1 precursor	129
Histidine –rich glycoprotein	59
Kininogen 1	48

Table 4. Potential serum-binding proteins of adiponectin. Modified from Wang Y, *et al*, *Proteomics* 2006. 6:3862-3870.

Waki et al. recently identified the enzyme leukocyte elastase as an enzyme capable of cleaving adiponectin in its collagenous domain resulting in a globular fragment of 18-25 kD (162). It is reported that trypsin can completely digest trimeric adiponectin, but not the LMW and HMW forms (136). Additionally, protease A “Amano” (obtained from *Aspergillus oryzae*) and proteinase K (a non-specific serine protease obtained from *Tritirachium album*) were identified as proteases that are capable of selectively digesting the trimeric forms, and both the LMW and trimeric forms, respectively, allowing for the specific identification of the HMW and LMW (protease A) and HMW (proteinase K) complexes within the blood. It is currently believed that these proteases digest the collagen domain of human adiponectin (37).

As previously discussed, increased visceral adiposity is associated with the development of many metabolic abnormalities such as insulin resistance, dyslipidemia, hypertension, and glucose intolerance (1). Obesity, especially visceral obesity, is considered a major risk factor for the development of type 2 diabetes and insulin resistance. One possible link between increased visceral fat and the development of insulin resistance and diabetes may be adiponectin. Studies suggest that serum

adiponectin concentrations are determined predominantly by visceral fat content (150). *In vivo* and *in vitro* studies demonstrate that adiponectin secretion is higher from visceral adipose tissue compared to subcutaneous adipose tissue (29; 128; 138). This is opposite the secretion pattern of another adipokine, leptin, which is secreted primarily from subcutaneous adipose tissue (138; 150).

Gene expression

Northern blot analysis demonstrated that adiponectin is encoded by the human Adipose Most Abundant Gene Transcript 1 (apM1) (115). The adiponectin gene spans 17kB on human chromosome 3q27 which has been identified as a susceptibility locus for diabetes (161). The gene consists of three exons and two introns, and apM1 mRNA has been detected in human adipose tissue, but not skeletal muscle, small intestine, placenta, uterus, kidney, liver, lung, brain or heart. Therefore, it is believed that apM1 gene is expressed exclusively in adipose tissue (115). The nucleotide sequence for adiponectin has been compared among several species, and exhibits a high degree of homology. The porcine sequence shares approximately 88, 86, 85, and 83% homology with dog, human, cow, and mouse adiponectin, respectively (86). The expression of apM1 is highly restricted to mature fat cells, and initial expression of the gene does not appear until approximately day four following induction of differentiation of preadipocytes into adipocytes (76; 115). It is not expressed in 3T3-L1 fibroblasts, however the gene is induced over 100-fold following differentiation of 3T3-L1 fibroblasts into 3T3-L1 adipocytes (147).

Specific mutations in the adiponectin gene have been observed to result in lower plasma levels of adiponectin. A G/T polymorphism has been reported in exon 2 and a

missense mutation (R112C) in exon 3 (153). Although the plasma levels of adiponectin in patients with the G/T polymorphism were low, they were not significantly different from plasma levels of adiponectin in normal patients (153). The one subject with the missense mutation (R112C) showed very low plasma levels of adiponectin suggesting the missense mutation was responsible for the low levels of the protein. Mondo *et al.* characterized 3 more missense mutations of the adiponectin gene in locations encoding the globular domain of the protein (104). These mutations included the previously described R112C as well as newly described I164T, R221S, and H241P. The frequency of the I164T mutation was significantly higher in type 2 diabetic patients than in age-matched and BMI-matched control subjects (104). Plasma levels of adiponectin in individuals carrying this mutation were also significantly lower than adiponectin levels in individuals without the mutation, and all patients identified with this mutation had at least one feature of the metabolic syndrome such as hypertension, hyperlipidemia, diabetes or atherosclerosis (104). These results give further evidence to support the role of adiponectin in the development of type 2 diabetes or other components of the metabolic syndrome.

Adiponectin receptors

In 2003, Yamauchi *et al.* cloned the human and mouse adiponectin receptors designated AdipoR1 and AdipoR2 through a series of expression cloning experiments. The gene for the human AdipoR1 receptor is located on chromosome 1p36.13-q41, whereas the gene for AdipoR2 on 12p13.31. The mouse genes for AdipoR1 and AdipoR2 are located on chromosomes 1 E4 and 6 F1, respectively. Mouse AdipoR1 encodes a protein of 375 amino acids with a predicted molecular mass of 42.4 kD and mouse AdipoR2 encodes a protein of 311 amino acids with a predicted molecular weight of 35.4

kD. Although these receptors are expressed ubiquitously, AdipoR1 is more highly expressed in skeletal muscle and AdipoR2 is more highly expressed in the liver. Scatchard plot analyses showed that AdipoR1 is a high-affinity receptor for the globular domain of adiponectin but a very low-affinity receptor for full length adiponectin, and that AdipoR2 is an intermediate affinity receptor for globular domain and full length adiponectin (172). The full-length form of the protein is believed to be the circulating form of adiponectin. The globular domain of the protein has been demonstrated to have more potent effects on fatty acid oxidation in skeletal muscle than the full-length protein (173). Recently, adiponectin receptors have been identified in other tissues and species. Fasshauer *et al.* demonstrated that AdipoR2 was expressed in 3T3-L1 adipocytes, indicating that adiponectin has autocrine characteristics (44). Kharroubi *et al.* demonstrated both AdipoR1 and AdipoR2 expression in pancreatic β cells of mice and humans (94). Most recently, AdipoR1 and AdipoR2 transcripts were found in heart, skeletal muscle, adipose tissue, liver, and spleen of pigs, with AdipoR1 being more abundantly expressed in heart and skeletal muscle and AdipoR2 in adipose tissue (35).

Both receptor subtypes are predicted to contain seven-transmembrane domains, however they are functionally and structurally distinct from G-protein coupled receptors (172). The N-terminus of the adiponectin receptor lies on the internal surface of the cell membrane while the C-terminus is on the external surface of the cell membrane. This is opposite the structural topology of typical G-protein coupled receptors (172). Although the full signal transduction pathway upon adiponectin binding to its receptor is currently unknown, it has been reported that binding of globular and full-length adiponectin to AdipoR1 or AdipoR2 increases PPAR- α ligand activity, and increases the

phosphorylation of adenosine monophosphate protein kinase (AMPK), acetyl coenzyme A carboxylase (ACC), and p38 mitogen-activated protein kinase (p38 MAPK). These events result in increased fatty acid oxidation and increased glucose uptake in skeletal muscle (AdipoR1) and decreased glucose output by the liver (AdipoR2), accounting for increased insulin sensitivity (172).

Recent *in vivo* and *in vitro* studies have investigated whether AdipoR1 and AdipoR2 expression levels are altered in pathophysiological states. Civitarese *et al.* suggested that AdipoR1 and AdipoR2 expression levels in muscle are lower in subjects with a family history of type 2 diabetes than those without (27). Importantly, the expression levels of both receptors correlated positively with insulin sensitivity. In contrast to these results, Debard *et al.* did not demonstrate any significant differences in gene expression levels of AdipoR1 or AdipoR2 in skeletal muscle of type 2 diabetic patients (33). Staiger *et al.* also investigated the expression levels of AdipoR1 and AdipoR2 from human myotubes and found that neither AdipoR1 nor AdipoR2 correlated with insulin sensitivity (149). In experiments utilizing transgenic mice in which native full length adiponectin was placed under the control of the adipocyte promoter aP2 such that it was moderately expressed from adipose tissue, adiponectin mRNA from fat depots and circulating concentrations were reduced. Concomitantly, the expression of AdipoR2 was downregulated while AdipoR1 was unaffected. These experiments provide evidence of a regulatory feedback loop by which adiponectin downregulates its own production and the expression of its AdipoR2 receptor (6). It is clear that further investigation of the expression of AdipoR1 and AdipoR2 will be necessary to elucidate whether there is indeed a correlation between adiponectin receptor levels and insulin sensitivity.

Tsuchida *et al.* recently reported that AdipoR1 and AdipoR2 expression levels are under nutritional control. In the fasted state, AdipoR1 and AdipoR2 expression increased in both liver and skeletal muscle of mice compared to mice fed *ad libitum* (158). Upon refeeding, AdipoR1 and AdipoR2 expression levels decreased in both liver and skeletal muscle. They also demonstrated that adiponectin receptors are negatively regulated by insulin (158). Insulin regulation of AdipoR1 and AdipoR2 was suppressed by the PI3-kinase inhibitor LY294002 suggesting that insulin regulation of AdipoR1 and AdipoR2 occurs through the PI3-kinase pathway (158).

AdipoR1 and AdipoR2 expression was investigated in skeletal muscle and liver from two different models of diabetic mice. Expression of AdipoR1 and AdipoR2 was increased in the skeletal muscle of streptozotocin (STZ) diabetic mice (158). Treatment of STZ mice with insulin reduced the expression levels of AdipoR1 and AdipoR2. Inukai *et al.* also showed that AdipoR1 expression was increased in STZ treated mice and that gene expression and protein concentrations were reduced with insulin treatment. However, AdipoR2 mRNA concentrations in STZ or STZ + insulin treated mice in these studies were not significantly changed when compared to controls. Expression of AdipoR1 and AdipoR2 was not significantly different in the livers of STZ diabetic mice compared to non-STZ mice, however insulin significantly decreased expression of both receptors in the livers of STZ treated mice (158). AdipoR1 and AdipoR2 expression levels were reduced in muscle and adipose tissue but not liver of *ob/ob* mice, a model commonly used to study type 2 diabetes. They also suggest that downregulation of adiponectin receptors in *ob/ob* mice is correlated with decreased adiponectin sensitivity (158). In *db/db*, genetically obese mice that exhibit overt hyperglycemia and

hyperinsulinemia, AdipoR1 expression was decreased, which is contrary to that which would be expected, suggesting that another mechanism, which is dominant over insulin signaling, regulates AdipoR1 gene expression (80).

In 2004, utilizing expression cloning, Hug *et al.* reported the existence of another adiponectin receptor called T-cadherin (78). It is a member of the cadherin family of receptors, which are generally involved in calcium mediated cell-cell interactions, and is structurally distinct from AdipoR1 and AdipoR2. With the exception of T-cadherin, all members of the cadherin superfamily contain a transmembrane domain, linking the extracellular portion of the molecule with the intracellular signaling pathways. The extracellular portion of T-cadherin receptor contains five ectodomains, with ectodomain-1 being implicated as integral to cellular functions. The C-terminus contains a GPI anchor that binds lipoproteins. T-cadherin was initially described in the nervous system, but its tissue distribution is widespread with the highest expression in the cardiovascular system and lower expression in muscles. T-cadherin is expressed in smooth muscle cells as well as endothelial cells and binds eukaryotically-produced high molecular weight as well as LMW complexes of adiponectin, but not the trimeric or globular forms of adiponectin. T-cadherin does not bind bacterially produced adiponectin suggesting that post-translational modifications are necessary for the binding of adiponectin to the T-cadherin receptor (78).

Physiological functions of adiponectin

A wide array of physiological functions in a variety of tissues including skeletal muscle, liver and the vasculature occur following adiponectin binding to its receptors. Adiponectin has been demonstrated to have anti-inflammatory, anti-atherogenic, and

anti-proliferative properties (163). The globular C-terminal domain and the trimeric forms of adiponectin activate AMPK and lead to increased fatty acid oxidation and reduction of serum glucose by several mechanisms, whereas the LMW and HMW forms of adiponectin activate NF- κ B pathways and have been shown to inhibit apoptosis of endothelial cells (103). Studies showed that adiponectin suppressed activation of the transcription factor, NF- κ B, in the vascular endothelium (135). TNF- α regulates the activation of NF- κ B, which results in the accumulation of cAMP. This activation is blocked by adenylyl cyclase and protein kinase A (PKA) inhibitors suggesting that adiponectin mediates this inflammatory response through the cAMP-PKA and NF- κ B pathways (135). Adiponectin was also shown to adhere to injured vascular walls, preventing the adhesion of macrophages as well as plaque formation (134). Interestingly, diabetic patients with coronary artery disease (CAD) had lower concentrations of adiponectin than diabetic patients without CAD (74), suggesting that adiponectin deficiency may play a role in the development of CAD. Adiponectin has been shown to inhibit proliferation of aortic smooth muscle cells, myelomonocytic cells, endothelial cells, and hepatic stellate cells. Adiponectin inhibits atherogenesis by accumulating in the subintimal space of injured vessels, interacting with collagens in the vascular intima and inhibiting TNF- α induced monocyte adhesion and expression of E-selectin, VCAM-1, and ICAM-1 (40). There is also evidence that adiponectin selectively binds with several mitogenic growth factors that can induce cell proliferation in many types of cells. The interaction of adiponectin with these growth factors can preclude their binding to the membrane receptors and lead to the attenuation of their mitogenic activity, suggesting that the anti-proliferative effect of adiponectin is at least partly due to its selective

sequestration of growth factors at a pre-receptor level. Additionally, adiponectin has been found to bind growth factors based on its oligomeric form. For instance, platelet-derived growth factor BB (PDGF-BB) binds to the HMW and LMW, but not to the trimeric form of adiponectin. Basic fibroblast growth factor (FGF) preferentially interacts with the HMW form, and heparin-binding epidermal growth factor-like growth factor (HBEGF) binds to the HMW, LMW, and trimeric forms with comparable affinities (163).

Adiponectin may also be an important link between obesity, insulin resistance and the development of type 2 diabetes. Adiponectin concentrations are reduced in obese adults as well as adults with type 2 diabetes (74; 123). More recent studies suggest that decreased adiponectin concentrations are more strongly correlated with insulin resistance and hyperinsulinemia than degree of adiposity and glucose tolerance (151; 169). Low basal concentrations of adiponectin in a population of Pima Indians were found to predict decreased insulin sensitivity in this population regardless of adiposity (110). In addition, adiponectin concentrations are positively correlated with high-density lipoprotein levels (HDL); low concentrations of adiponectin are associated with high levels of low density lipoproteins (LPL) as well as other metabolic parameters involved in the development of the metabolic syndrome (59). These studies point to a role of adiponectin in increasing insulin sensitivity and suggest decreases of this hormone are associated with decreased insulin sensitivity.

There is an abundant amount of experimental evidence supporting the role of adiponectin as an insulin-sensitizing hormone. Intraperitoneal (ip) injection of adiponectin lowered serum glucose levels and free fatty acids without changes in insulin

levels in mice (8; 52). Chronic injections of adiponectin resulted in decreased body weight in high-fat-fed mice (52) and improved insulin sensitivity in insulin resistant mice (174). Interestingly, injections of the isolated C-terminal globular domain had the same, if not more potent, metabolic effects than the full-length protein (8; 52; 174). Recently, Pajvani *et al.* showed that the ratio, and not the absolute amounts, between the two oligomeric forms of adiponectin (HMW to LMW) are critical in determining insulin sensitivity. They defined a new index, S_A , that can be calculated as the ratio of $HMW/(HMW+LMW)$, - serving as a quantitative indicator of improvement of insulin sensitivity. Compared to total adiponectin, the S_A ratio and 2 hour glucose concentrations from an oral glucose tolerance test displayed a strong linear relationship, suggesting that S_A could act as a better marker of glucose intolerance (50). In male mice, the majority of adiponectin is present as the LMW form, whereas female mice have a more even distribution of complexes. The HMW form selectively disappears (with no increase in the LMW form) in mice treated with insulin or glucose, suggesting that HMW adiponectin complexes circulating in serum represent a precursor pool that can be activated by metabolic stimuli and subsequently dissociate into a transient bioactive trimer (136). Alternatively, another research team suggests that it is the quantity of HMW, not total or HMW-to-total adiponectin (S_A) ratio that is responsible for adiponectin's association with increased insulin sensitivity, reduced abdominal fat, and high basal lipid oxidation (106).

Skeletal muscle is a major target for adiponectin's physiological functions. Improved insulin sensitivity may be the result of increased fatty acid oxidation and decreased triglyceride content in muscle (52; 174). Yamauchi *et al.* demonstrated that

globular and full-length adiponectin phosphorylated and activated AMPK in skeletal muscle (173). Activation of AMPK resulted in phosphorylation of acetyl coenzyme A carboxylase, (ACC), fatty acid oxidation, glucose uptake and lactate production in C2C12 myocytes.

Liver is another primary target organ of adiponectin's insulin sensitizing properties. Adiponectin enhanced insulin action in primary hepatocytes by decreasing gluconeogenesis and hepatic glucose output (8). However, exposure to the isolated globular C-terminus did not have the same metabolic effects in isolated hepatocytes, indicating that the full-length protein is necessary for adiponectin's insulin-sensitizing effects in the liver (8). Adiponectin mediates these insulin-sensitizing effects by increasing fatty acid oxidation through activation of AMPK and PPAR- α (173). Activation of AMPK in the liver is followed by increased phosphorylation of ACC, decreased malonyl-CoA and increased fatty acid oxidation (173). Activation of PPAR- α also results in increased gene expression of enzymes involved in fatty acid oxidation (49). The expression levels of enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) involved in gluconeogenesis were decreased in mice infused with Acrp30 (32). These results suggest that adiponectin lowers circulating glucose levels by decreasing hepatic glucose output (32).

Qi *et al.* recently demonstrated that adiponectin is present and has effects in the brain (140). Their results suggest that adiponectin is transported from the serum to the cerebral spinal fluid (CSF). Intracerebroventricular (ICV) injections of adiponectin in normal mice reduced the body weight of these mice without affecting food intake. Increased oxygen consumption as well as increased expression of uncoupling protein-1

(UCP-1) in brown adipose tissue was also observed in these mice. These results suggest that ICV injections of adiponectin increased thermogenesis in these animals.

Importantly, ICV injections of adiponectin in Agouti mice did not have any effects on body weight or thermogenesis. This suggests that these effects of adiponectin are mediated at least in part through the melanocortin pathway (140). Figure 3 represents a hypothetical model for the actions of adiponectin in its target tissues including liver, skeletal muscle, brain and the endothelium (5).

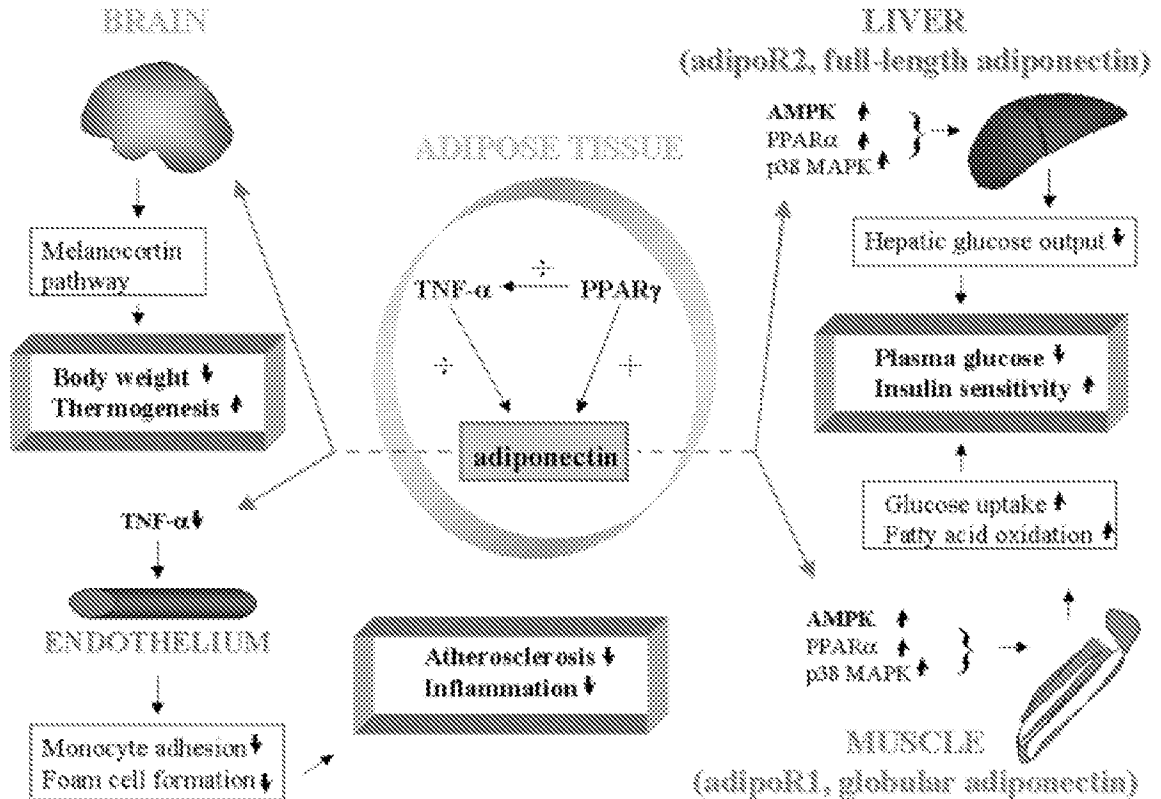


FIGURE 3: Illustration of target tissues and proposed mode of action and regulation of adiponectin. From Lihn A. S. *et al. Obesity Reviews.* 2005. 6, 13-21.

Further characterization of adiponectin as an insulin-sensitizing hormone has been undertaken using genetically modified or knockout mice. However, these studies have produced conflicting data. Maeda *et al.* reported that mice lacking a functional adiponectin gene and fed a normal diet did not show any differences in growth rate, food intake, body weight or metabolic parameters measured at 12 and 16 weeks of age compared to wild type mice (116). However, these knockout mice did show delayed clearance of free fatty acids in plasma, low levels of fatty acid transport protein-1 (FATP-1) mRNA in muscle as well as high levels of TNF- α mRNA in adipose tissue and high

circulating levels of TNF- α (116). Additionally, knockout mice fed a high fat/high sucrose diet for two weeks had significantly higher plasma glucose levels and insulin concentrations compared to wild type mice. Six weeks on the high-fat/high-sucrose diet did not result in increased body weight or adiposity compared to wild type mice. However, it did result in the development of severe insulin resistance (116). Adenovirus-mediated increased adiponectin expression in the knockout mice reversed the reduction of FATP-1 as well as the increase in TNF- α and the diet-induced insulin resistance. In a subset of these studies, mice were also generated heterozygote for adiponectin expression. No significant differences in any of the metabolic parameters measured as well as insulin sensitivity were recorded between these mice on either the normal diet, or the high-fat/high-sucrose diet compared to wild type mice (116).

Ma *et al.* also generated adiponectin knockout mice. Their results, however, were quite different from those reported by Maeda *et al.* Adiponectin deficient mice were found to have normal body and fat pad weights as well as normal circulating glucose and insulin levels (113). They also responded to a glucose tolerance test in a manner similar to wild type mice suggesting no overt insulin resistance. High fat feeding for seven months did not result in any significant differences in weight gain or glucose or insulin tolerance tests between normal and adiponectin deficient mice (113). Increased β -oxidation in skeletal muscle and liver was the only significant difference found between the adiponectin knockout mice and wild type (113). The reasons for the conflicting results between these studies are unclear however a number of possibilities exist, including differences in the generation of the knockout mice as well as the duration and composition of the diet.

Regulation of adiponectin

Due to adiponectin's important insulin-sensitizing, anti-atherogenic and anti-inflammatory properties, it has become of major interest to identify factors that play a role in the regulation of adiponectin expression and secretion from adipose tissue. Identification of factors involved in the regulation of adiponectin secretion and expression may lead to the identification of potential therapeutic targets aimed at elevating levels of this protein in disease states such as coronary artery disease and type 2 diabetes.

Adiponectin concentrations are influenced by many factors. Scherer *et al.* were the first to describe that insulin acutely stimulates secretion of adiponectin from 3T3-L1 adipocytes (146). Longer term incubation (>16 hr) with insulin, however, has been shown to increase or decrease both adiponectin secretion and gene expression (67). Interleukin - 6 and TNF- α are cytokines produced from adipose tissue, and levels of these cytokines are increased in insulin resistance and obesity. Both cytokines inhibit adiponectin gene expression and secretion from adipocytes (45; 46). Table 5 provides a brief summary of some of the factors involved in the regulation of adiponectin secretion and gene expression.

Other hormonal regulatory factors of adiponectin include insulin-like growth factor (IGF-1), growth hormone, and leptin. IGF-1 increases adiponectin gene expression in adipocytes. Growth hormone increases adiponectin gene expression and secretion. Leptin, another adipokine whose levels are dysregulated in insulin resistance, obesity and type 2 diabetes, has been shown to inhibit adiponectin secretion.

Pharmacological agents have also been shown to affect adiponectin gene expression and protein secretion. PPAR γ ligands such as the anti-diabetic class of drugs thiazolidinediones (TZDs) are known to enhance insulin sensitivity in insulin resistant type 2 diabetic animal models as well as insulin resistant patients. Previously, it was unknown how the TZDs increased insulin sensitivity, however recent studies have shown that TZDs increase adiponectin expression and secretion from adipocytes. Therefore, it is believed that at least a portion of the insulin-sensitizing properties of the TZDs results from increased circulating levels of adiponectin.

FACTOR	ADIPONECTIN GENE EXPRESSION	ADIPONECTIN SECRETION
Insulin	+/-	+/-
IGF-1	+	Not determined
PPAR γ ligands/TZDs	+	+
TNF- α	-	-
Glucocorticoids	-	Not determined
β -adrenergic agonists	-	-
cAMP	-	-
Interleukin-6	-	-
Growth Hormone	+	+
Exercise	Not determined	+
Leptin	Not determined	-
Free Fatty Acids	Not determined	-
Weight loss	+	+

Table 5. Factors influencing adiponectin secretion and gene expression. Modified from Stefan and Stumvoll. *Hormone and Metabolic Research*. 2002.34:469-474.

Interestingly, there appear to be gender-related differences in the circulating concentrations of adiponectin. Plasma adiponectin levels are 35% lower in human males compared to human females (6.0 $\mu\text{g/ml}$ vs. 9.1 $\mu\text{g/ml}$) (133). There are no significant differences in plasma adiponectin levels between pre- and post-menopausal women (133). This suggests that male sex hormones are a potential regulator of circulating adiponectin levels. Indeed, Nishizawa *et al.* demonstrated that testosterone decreased

adiponectin secretion from 3T3-L1 adipocytes, and castration of male mice increased circulating adiponectin levels compared to non-castrated littermates (133).

Adiponectin has a relatively long half-life in serum (2.5-6 h) (73; 136), and it was recently determined that adiponectin levels are also determined in part by ultradian patterns. In other words, adiponectin is secreted with diurnal variations in less than a 24 hour period (54). In normal human males, serum adiponectin was characterized by a nocturnal decline starting in the late evening and reaching its maximum decrease in the early morning (~ 3am; $3.56 \pm 0.3 \mu\text{g/ml}$) (54). Serum adiponectin levels were higher during the day with peak levels reached at approximately 11am ($5.28 \pm 0.3 \mu\text{g/ml}$) (54). Although both adiponectin and leptin exhibit similar variations in diurnal rhythms, these rhythms do not overlap, and suggest a possible regulation by one adipokine of the other. Indeed, a recent study suggests that leptin plays a role in the regulation of circulating adiponectin levels. Central leptin gene therapy administered in *ob/ob* and wild type mice decreased plasma levels of adiponectin without an alteration in TNF- α levels (159). In addition, peripheral administration of leptin also significantly reduced plasma levels of adiponectin in *ob/ob* and wild type mice (159).

The effects of weight loss and exercise have also been investigated as potential mediators of adiponectin secretion and expression. Obese men (BMI $>35 \text{ kg/m}^2$) were placed on a calorie restricted diet for 20 weeks. Prior to weight loss, plasma adiponectin levels were 47% lower in obese mean compared to lean individuals (18); following diet-induced weight loss of approximately 20kg, plasma adiponectin levels increased by 51% (2.3 ± 0.6 vs. $3.4 \pm 0.8 \text{ mg/l}$). Adiponectin mRNA was collected from adipose tissue biopsies from these patients before and after weight loss, and following weight reduction,

adiponectin mRNA levels increased by 45% (18). Plasma adiponectin was also measured in a group of individuals before and after weight loss by gastric partition surgery. Prior to surgery the patients had a mean BMI of $39.57 \pm 5.89 \text{ kg/m}^2$ and an average circulating concentration of adiponectin of $4.53 \pm 1.46 \mu\text{g/ml}$ (175). Following gastric partitioning surgery, the subjects lost >20kg, had an average BMI of $31.22 \pm 5.21 \text{ kg/m}^2$, and average circulating concentration of adiponectin of $6.63 \pm 2.32 \mu\text{g/ml}$ (175). In both of these studies, the increases in plasma adiponectin levels were significantly correlated with increases in insulin sensitivity (18; 175).

Other studies provide further evidence for the dysregulation of adiponectin in obesity and diabetes. Hu *et al.* demonstrated that apM1 gene expression is dysregulated in obesity. A reduction in apM1 mRNA was observed in both obese humans and mice as compared to normal controls (76). Arita *et al.* measured plasma adiponectin from non-obese and obese subjects and found that circulating levels of this protein are significantly reduced in obese subjects ($8.9 \mu\text{g/ml}$ vs. $3.7 \mu\text{g/ml}$) (2). Thiazolidiones (TZDs) such as rosiglitazone and pioglitazone, are a class of anti-diabetic drugs which have been shown to improve insulin sensitivity. Administration of TZDs to obese mice and cultured 3T3-L1 adipocytes increased mRNA expression of apM1 in a dose- and time-dependent manner (117). Administration of TZDs to insulin-resistant humans and mice also increased circulating levels of adiponectin (117).

Adiponectin and Adiponectin Receptors in Other Species

Adiponectin has been identified in several other species including non-human primates, pigs, yellow-bellied marmots, blue foxes, and dogs. Many rhesus monkeys (*Macaca mulatta*) spontaneously develop obesity and subsequently develop type 2

diabetes mellitus, thus making an excellent animal model for human type 2 diabetes mellitus. Resembling humans, plasma adiponectin concentrations are decreased in rhesus monkeys with obesity and in those with type 2 diabetes. Plasma concentrations of adiponectin begin to decrease in the earliest stage of obesity, when insulin resistance and hyperinsulinemia are progressing, and before the onset of type 2 diabetes mellitus (75).

In 2004, Jacobi *et al.* reported the porcine adiponectin ORF and provided the amino acid sequence inferred from the nucleotide sequence. The porcine nucleotide sequence shares approximately 88, 86, 85, and 83% homology with dog, human, cow, and mouse adiponectin, respectively, and 79-83% similarity with dog, human, cow, and mouse proteins at the amino acid level. Lower plasma concentrations of adiponectin are associated with greater adiposity in the pig and the difference is established relatively early in the growth curve (approximately 90 days of age). Additionally, *in vitro* studies show that intact adiponectin acts directly upon porcine adipocytes to suppress lipogenesis. Relative plasma adiponectin concentrations are not altered in pigs infused with *E. coli* and mRNA expression in adipose tissue is not responsive to lipopolysaccharide (LPS) infusion, indicating that expression and secretion of adiponectin are not acutely regulated *in vivo* to alter the circulating protein concentration during infection (86). Adiponectin suppresses both TNF α and IL-6 production in porcine macrophages activated with LPS and increases IL-10 expression and augments the induction of this cytokine by LPS. The anti-inflammatory activity of adiponectin is achieved in part by inhibition of NF κ B activation and suppression of ERK1/2 activity (170). The pig is emerging as a valuable research model to evaluate the effects that adiponectin has on adiposity, atherogenesis, and inflammation.

The AdipoR1 and AdipoR2 receptors have been cloned from pig tissues and possess a similar predicted amino acid sequence as those of the human and mouse, ranging from 81-97% homology. Such a high degree of homology suggests that porcine AdipoR1 and AdipoR2 have similar functions as those described in other species. Transcripts for AdipoR1 were abundant in heart and skeletal muscle and also detected to a lesser extent in adipose tissue, liver, and spleen, whereas transcripts for AdipoR2 were abundant in subcutaneous adipose tissue and present to a lesser extent in the liver, heart, skeletal muscle, and spleen. In contrast, AdipoR2 in mice is most highly expressed in the liver, indicating that a species difference exists between mice and pigs. Porcine adipose tissue is the major lipogenic tissue, with high lipolytic activity. The greater AdipoR2 mRNA concentration in porcine adipose tissue indicates that AdipoR2 plays a more significant role in enhancing glucose import for lipogenesis and fatty acid oxidation in the adipose tissue than other tissues. Short term fasting (8 hours) does not affect AdipoR1 mRNA concentration, whereas AdipoR2 mRNA concentration is increased in the adipose tissue of food-restricted pigs (35).

Yellow-bellied marmots (*Marmota flaviventris*) are hibernators that represent an interesting and valuable animal model to study adiponectin's effect on energy balance and regulation of fat mass. Yellow-bellied marmots increase their food intake prior to hibernation and dramatically increase body mass and specifically fat mass, which is used as the primary metabolic fuel while fasting during hibernation. Plasma adiponectin concentrations generally decrease from summer to winter and adiponectin mRNA levels were significantly higher in summer compared to autumn and winter (51).

The blue fox (*Alopex lagopus*) is a farm-bred variant of the arctic fox, which is a middle-sized carnivore with an arctic circumpolar distribution. The farmed blue fox is a domesticated type of the blue fox descending from the Alaskan and Greenland arctic foxes. Arctic foxes have adapted to the extreme environment of the high arctic, where it is essential to assimilate every bit of available energy as efficiently as possible. The blue fox has retained the ability to deposit a large amount of white adipose tissue (subcutaneous and intraabdominal) in the autumn to be mobilized in the winter and during the vernal reproductive season. Wild arctic foxes can experience involuntary fasts for several days until a source of nutrition is found. During fasting a wide array of weight-regulatory hormones controls the mobilization and conservation of energy stores in the farmed blue fox and the wild arctic fox, enhancing the changes of an individual to survive through nutritional scarcity. During a 22 day fast, adiponectin plasma concentrations, which ranged from 3-12 $\mu\text{g/mL}$, were found to be 24-48% higher than those of the fed controls between days 8-22, but acutely (48 hours) had no effect on adiponectin concentrations. The raccoon dog (*Nyctereutes procyonoides*), which is an omnivorous canid that utilizes passive wintering strategy, exhibits a decrease in plasma adiponectin concentrations under food deprivation. As adiponectin is known to reduce hepatic gluconeogenesis, it can be hypothesized that increased adiponectin concentrations during fasting could aggravate hypoglycemia potentially detrimental to wintering animals. The raccoon dog is able to sustain stable plasma glucose concentrations during long periods of food deprivation. Decreased adiponectin concentrations could contribute to effective gluconeogenesis, keeping the animals alert and capable of foraging or reacting rapidly to danger (132). Adiponectin concentrations did not respond to

wintertime fasting (7 days) in the American mink (*Mustela vison*) (130). The blue fox, with its profound seasonal cycles of body fat gain and loss and the raccoon dog, with its ability to gather extensive fat reserves in the autumn leading to nonpathological obesity are especially fascinating models to study the physiological roles of metabolic hormones (129).

Unlike mammals, the adiponectin gene is expressed in several tissues in the chicken, including adipose, liver, anterior pituitary, diencephalon, kidney, skeletal muscle, ovary, and spleen. Adipose tissue, liver, anterior pituitary, diencephalon, kidney, and skeletal muscle were found to contain the highest amount of adiponectin mRNA, respectively. The open reading frame of chicken adiponectin is 735 bp and yields a protein of 244 amino acids. Chicken adiponectin cDNA is 65-85% homologous to pig, human, mouse, or dog adiponectin cDNA and the deduced protein sequence is 51-61% similar to mammalian adiponectin. Food deprivation results in a significant decline in adiponectin mRNA quantity in adipose tissue, liver, and anterior pituitary gland, indicating that adiponectin gene expression is closely linked to energy balance in these tissues (114). AdipoR1 and AdipoR2 have recently been cloned in chickens and the cDNA open reading frames consist of 1128 and 1161 bp, respectively. Chicken AdipoR1 and AdipoR2 cDNA sequences are only 68% homologous to each other, however AdipoR1 cDNA was found to be 80-83% homologous to human, mouse, rat, or pig with a deduced protein sequence 91% similar to mammalian species and AdipoR2 cDNA was found to be 76-78% homologous to the same species with a deduced protein sequence 82% similar to mammalian species. Skeletal muscle, adipose tissue, and diencephalons contain the highest quantities of AdipoR1 mRNA followed by kidney, ovary, liver,

anterior pituitary, and spleen. Adipose tissue has the highest quantity of AdipoR2 mRNA followed by skeletal muscle, liver, ovary, diencephalons, anterior pituitary, kidney, and spleen. Forty eight hour feed deprivation causes increased AdipoR2 mRNA quantity in adipose tissue and decreased AdipoR1 mRNA quantity in the anterior pituitary gland with no change in the liver and the diencephalons. The determination of the metabolic effects of adiponectin and its receptors in the chicken will provide interesting information in that this species has physiologically higher concentrations of blood glucose compared to mammals (141).

Hypothesis and Objectives

The metabolic derangements associated with obesity and diabetes have recently been linked to changes in circulating concentrations of cytokines secreted from adipose tissue (adipokines). Most notable among these secreted proteins is adiponectin, whose concentrations are increased in type 1 diabetes and decreased in both obesity and type 2 diabetes, possibly providing a link between the latter two pathophysiological conditions (86). The central hypothesis of the proposed research is that adiponectin is a critical player in metabolic abnormalities associated with obesity and diabetes in the dog. We hypothesize that adiponectin is present within canine serum and circulates as similar protein complexes as those described in the human and its secretion pattern is altered in the disease states of obesity and insulin resistance.

The purpose of the present studies is to characterize adiponectin protein complex formation, secretion, gene expression, and stability in normal and diseased (nutritionally obese/insulin resistant) dogs. Our first series of studies will characterize adiponectin in normal dogs. Our second series of studies will investigate the pathophysiologic impact

of canine obesity and insulin resistance on adiponectin secretion (including protein multimer profiles and serum concentrations) and gene expression. At the end of these studies, we will provide evidence that adiponectin is secreted as protein complexes that resemble the pattern observed in humans. We will also provide evidence that the disease states of canine obesity and insulin resistance alter this secretion pattern as well as the serum concentrations and gene expression patterns of adiponectin. In establishing a correlation between adiponectin's secretion pattern and nutritional obesity/insulin resistance in dogs, we may further establish a link between obesity, the development of insulin resistance and possibly type 2 diabetes mellitus, as well as offer a clinical marker for the early detection of these conditions in a clinical setting.

CHAPTER III.

CHARACTERIZATION OF ADIPONECTIN IN THE DOG

Abstract

Objective-To assess serum concentrations of adiponectin and characterize adiponectin protein complexes in healthy dogs.

Animals-11 healthy dogs

Procedure-Sera collected from 10 dogs were evaluated via velocity sedimentation and ultracentrifugation, SDS-PAGE, Western immunoblotting, and radioimmunoassay.

Visceral adipose tissue (approximately 90 grams) was collected from the falciform ligament of a healthy dog undergoing elective ovariohysterectomy, and adiponectin gene expression was assessed via a real-time PCR procedure.

Results- Adiponectin gene expression was detected in visceral adipose tissue. Serum adiponectin concentrations range from 0.85 $\mu\text{g/mL}$ to 1.5 $\mu\text{g/mL}$ (mean concentration, 1.22 $\mu\text{g/mL}$). In canine serum, adiponectin was present as a multimer, consisting of a low molecular weight complex (180 kD); as 3 (180-, 90-, and 60 kD) complexes under denaturing conditions; as 2 (90- and 60 kD) complexes under reducing

conditions; and as a dimer, a monomer, and globular head region (60, 30, and 28 kD, respectively) under reducing-denaturing conditions. It is likely that adiponectin also circulates as a high molecular weight (360- to 540-kD) complex in canine serum, but resolution of this complex was not possible via SDS-PAGE.

Conclusions and Clinical Relevance- After exposure to identical experimental conditions, adiponectin protein complexes in canine serum were similar to those detected in human and rodent sera. Circulating adiponectin concentrations in canine serum were slightly lower than concentrations in human serum. Adiponectin gene expression was identified in canine visceral adipose tissue. Results suggest that adiponectin could be used as an early clinical marker for metabolic derangements, including obesity, insulin resistance, and diabetes mellitus in dogs.

Introduction

Obesity, which is defined as an accumulation of excess body fat, is the most common nutritional disorder in small animals and is associated with various diseases such as diabetes mellitus, pancreatitis, cardiovascular disease, arthropathies, and increased surgical risk (84). Although most dogs with diabetes mellitus are thought to have a disease that is similar to type 1 diabetes of humans and are insulin dependent, it has been proposed that diabetes in adult dogs progresses through several stages; these stages begin with impaired glucose tolerance, followed by non-insulin dependent stages resembling type 2 diabetes in humans, and finally progressing to insulin dependent diabetes mellitus (48). Obesity often results in the development of insulin resistance. In a recent study, it was determined that optimum glucose tolerance and insulin response appear to be integrally involved in the health and longevity of dogs (107); it was

suggested that insulin resistance in dogs has a similar etiology and consequence for health as it does in humans. However, dogs are most often evaluated by their veterinarian only when the owner suspects a problem, thus allowing insulin resistance to remain undetected (81). The link between obesity and the development of insulin resistance has yet to be elucidated. In humans and rodents, results of substantial research have indicated that adipose tissue is not simply an energy storage organ, but is a secretory organ that produces a variety of proteins that influence not only the metabolism of the body, but also endocrinologic and immunologic functions (2; 7). These metabolically active secretory products termed “adipocytokines”, include leptin, tumor necrosis factor- α (TNF α), adiponin, resistin, interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), and adiponectin (Acpr30) (26; 34). The role these adipocytokines play in the development of insulin resistance is being studied primarily in humans and rodents, with few studies having been performed to investigate their effects in dogs.

Adiponectin is an adipocytokine produced exclusively by white adipose tissue. It was identified independently by 4 groups (76; 115; 131; 147) and is known to be abundantly secreted into human plasma (accounting for 0.01% to 0.03% of total plasma protein) (139). Adiponectin has been established as an insulin sensitizer of the entire body because the globular C-terminal fragment of adiponectin is able to decrease plasma glucose concentrations and increase fatty acid oxidation in muscle and the full length fragment augments insulin-induced inhibition of glucose output in hepatocytes (47; 112). Even though our understanding of both type 1 and type 2 diabetes mellitus has greatly improved, the exact underlying pathologic mechanism leading to insulin resistance in these conditions has remained elusive. Compared with clinically normal humans, serum

adiponectin concentrations are decreased in humans that are obese or have type 2 diabetes; therefore, it is believed that decreased serum adiponectin concentrations links these two conditions and promotes the development of insulin resistance in humans through the regulation of glucose homeostasis and hepatic insulin sensitivity. Serum adiponectin concentrations are similarly decreased from clinically normal values in rodents and non-human primates that are obese or have type 2 diabetes (75). Surprisingly, adiponectin concentrations are increased in humans with type 1 diabetes (79).

Adiponectin circulates as three discrete protein complexes in the sera of mice and humans: a 90 kD trimer, a 180 kD LMW complex (also called a hexamer), and a higher order complex (>360 kD) comprised of 4 to 6 trimers termed the HMW complex. In male mice, most adiponectin is present as the LMW complex, whereas in female mice, there is a more even distribution of the LMW and HMW complexes. The HMW complex selectively disappears (with no increase in the LMW complex) in mice treated with insulin or glucose, suggesting that HMW adiponectin complexes circulating in serum represent a precursor pool that can be activated by metabolic stimuli and subsequently dissociate into a transient bioactive trimer (136). Recently, Pajvani *et al.* showed that the ratio, and not the absolute amounts, of the two oligomeric forms of adiponectin (HMW to LMW) is critical in determining insulin sensitivity. They proposed a new index, called the S_A index, that is calculated as the ratio of $HMW/(HMW+LMW)$, - and can be used as a quantitative indicator of change in insulin sensitivity.

In addition to humans, adiponectin has been identified in several other species, including rats, mice, dogs, and non-human primates. Adiponectin is highly homologous among species (rat:mouse, 92%; dog:mouse, 84%)(83). Serum adiponectin

concentrations have been investigated in yellow-bellied marmots (51) in relation to changes in lipid mass during hibernation and in fasting and fed arctic blue foxes(129). Adiponectin and its receptors (AdipoR1 and AdipoR2) have been identified in pigs (35; 51; 83; 86; 129). Unlike the numerous investigations in humans and laboratory animals, adiponectin has not yet been extensively studied in companion animals (83).

The purpose of the study reported here was to assess serum concentrations of adiponectin and characterize adiponectin protein complexes in healthy dogs. Furthermore, the gene expression of adiponectin in visceral adipose tissue was evaluated. The hypotheses of this study were that adiponectin is present in canine serum as protein complexes that are similar in human serum and that serum adiponectin concentrations in clinically healthy dogs are similar to those in clinically healthy humans. In addition, we hypothesized that adiponectin was stable at various storage temperatures (-80°C and -20°C) and that this stability was independent of the type of sample (serum, plasma-EDTA, or plasma-heparin samples).

Materials and Methods

Multiple Alignment Sequence- Nucleotide sequences for mouse (accession number NM 009605), rat (accession number NM 144744), human (accession number NM 004797) and dog (accession number NM 001006644) were identified by use of the the National Center for Biotechnology Information (NCBI) GenBank nucleotide search engine, converted to their respective amino acid sequences by use of a basic local alignment search tool, and compared by use of protein information resource multiple alignment computer software provided by Georgetown University.

Samples- Blood samples (total volume, 5 mL) were obtained from 10 healthy, university-owned dogs (4 females and 6 males; body weight range, 9.3 to 29.5kg) of mixed breeds and various ages by jugular venipuncture; blood was collected without anticoagulant to provide serum samples and with EDTA or heparin to provide plasma-EDTA and plasma-heparin samples. Serum and plasma samples were also obtained from the Auburn University College of Veterinary Medicine Clinical Pathology Laboratory. Precautions were taken to collect, handle, and store samples intended for assessment of storage conditions on the stability of adiponectin in a similar manner. All samples were placed on ice immediately following collection. To obtain serum, blood samples collected without anticoagulant were centrifuged; serum samples were immediately stored appropriately at -20° or -80° C for a period of as long as 342 days. Adipose tissue was obtained from the falciform ligament of a healthy female dog during routine, elective ovariohysterectomy and stored in an aqueous, nontoxic tissue storage reagent. Of the sample analyses, those performed 8 days following collection were considered to provide baseline data because little protein degradation was expected to have occurred during that storage interval. All procedures were approved by the Auburn University Institutional Animal Care and Use Committee prior to initiation.

SDS-PAGE and Western Immunoblotting-One microliter of serum from each dog was solubilized in Laemmli sample buffer (1:10) in the absence or presence of 5% 2-mercaptoethanol. Samples were incubated at room temperature (approximately 20° to 25° C). Samples were subsequently denatured by boiling for 10 minutes. Proteins were separated by SDS-PAGE (10%) and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in Odyssey blocking buffer, incubated overnight

(approximately 17 to 20 hours) with murine anti-adiponectin polyclonal antibody^a (1:1500 dilution), washed, and incubated with a fluorescently labeled secondary antibody^b (1:20,000 dilution). Blots were evaluated by use of the Odyssey infrared imaging system.

Radioimmunosassay- For each dog, serum adiponectin concentration was measured by use of a commercially available murine radioimmunoassay kit^c that measures total circulating adiponectin complexes (i.e. HMW, LMW, and trimer). Five microliters of serum was diluted in 2,495 microliters of 1x assay buffer (1:500). Samples were centrifuged for 30 min at 2,500 xg. All samples were assayed in duplicate. The sensitivity of this assay was 1.0 ng/mL with a standard range of 0.78-100 ng/mL. The inter-assay and intra-assay coefficients were 9.5% and 9.7%, respectively. Parallelism of canine samples was determined by serial dilution of five canine serum samples with comparison of results to the generated standard curve. Additivity was determined by spiking canine serum samples with known concentrations of murine adiponectin and assessment of subsequent measurements.

Real time PCR - Approximately 90 mg of adipose tissue collected from the falciform ligament of a clinically normal dog was stored in *RNAlater*^d. The RNA from adipose tissue was extracted with *TRIzol*^e. (Invitrogen, Carlsbad, CA). One microgram of total RNA was reverse transcribed using *iScript cDNA Synthesis kit* with 100 U of *Superscript II Reverse Transcriptase*^f (Bio-Rad, Hercules, CA). Adiponectin mRNA expression was measured by use of quantitative real-time PCR (*iCycler iQ Real-Time PCR detection System*^g, Bio-Rad, Hercules, CA). Two microliters of each RT reaction was amplified in a 30 μ L PCR containing 200 μ M of each primer and SYBR Green Super

Mix^h (Bio-Rad, Hercules, CA). Samples were incubated in the iCycler for an initial denaturation at 95 °C for 3.0 min followed by 40 PCR cycles. Each cycle consisted of 95 °C for 10 s and 58 °C for 1 min. Oligonucleotide primers used for adiponectin (Accession No. AB 110099.1) were AATCTTCTACAATCTGCAAAACCAC (sense) and TCTCGTATCGGAACAGAAAGAACAT (antisense) and GAPDH (Accession No. NM007475) are ACAGTCAAGGCTGAGAACGG (sense) and CCACAACATACTCAGCACCAGC (antisense). SYBR Green I fluorescence emission was measured after each cycle. Adiponectin mRNA levels were normalized to GAPDH expression. Amplification of specific transcripts was confirmed initially through sequencing and subsequently by producing melting curve profiles (cooling the sample to 55 °C and heating to 95 °C with continuous measurement of fluorescence) during each real-time PCR run.

Statistical Analysis- The factorial treatment structure of the storage experiment consisted of storage temperature (-20°C, -80°C), storage method (as serum, plasma-EDTA, or plasma-heparin), and storage duration (8, 96, 173, 250, 342 days). Because of radioactive decay a new RIA kit was used to analyze adiponectin concentrations in serum and plasma samples at each storage time. Comparisons involving storage durations are thus confounded with differences among RIA kits and the data were treated as a series of 2-factor experiments. A further complication arose from the fact that the treatment combination of plasma in heparin stored at -20° C was eliminated for cost reasons. The final analysis thus comprised two parts: a 2² factorial of method (serum and plasma in EDTA) and storage temperature (-20°C and -80°C), and a single-factor analysis of storage method (serum, plasma-EDTA, plasma-heparin) at -80°C. The experiment was

analyzed as a randomized complete block design with dog ($r = 10$) as the random effect representing blocks. The block-temperature interaction was used as the error term for temperature, and the pooled block-temperature-method interaction served as the experimental error term to test the effects of storage method and the storage temperature-method interaction. All treatment effects and their interactions were considered to be fixed. Because some observations were missing and the correlation among samples within blocks (aliquots were taken from blood that was collected from a given dog at a single time), mixed-model ANOVA procedures as implemented in the SAS® procedure MIXED were used (111). For all statistical analyses, a value of $p \leq 0.05$ was considered significant

Results

Multiple Alignment Sequence-Mouse, rat, human, and dog adiponectin protein sequences were compared. Resultant sequences were highly homologous (mouse:dog, 85%; rat:dog, 83%; and human:dog, 87%) . Specific peptide sequences recognized by the utilized mouse polyclonal antiadiponectin antibody (18-32; 187-200) were examined. Peptide sequence 187-200 was identical among the species examined. Peptide sequence 18-32 was not identical among the species examined. (Figure 4)

Real Time PCR – Gene expression of adiponectin was analyzed with primers specifically designed for canine adiponectin. Adiponectin gene expression was detected in the adipose tissue obtained from the falciform ligament of a healthy dog. (Figure 5)

Radioimmunoassay Validation-The mouse adiponectin radioimmunoassay used in this study was validated by use of standard additivity and parallelism assays. Percent recovery from serum samples spiked with 0.1, 0.5, or 1.0 ng of murine adiponectin

(because canine adiponectin is not commercially available) ranged from 76%-96%. Recovery rates improved as the concentration of added protein increased. (Figure 6) Differences between expected and measured concentrations were 24%, 11%, and 4% for the samples spiked with 0.1, 0.5, and 1.0 ng of murine adiponectin, respectively. A parallelism assay illustrated lines of equal slope, indicating that there was no significant proportional analytical error within the dilutional ranges of 1:50 to 1:5000. (Figure 7) Inter-assay and intra-assay coefficients of variation were 9.5% and 9.7%, respectively.

Radioimmunoassay of Canine Serum-Radioimmunoassay of sera from ten healthy dogs revealed circulating adiponectin concentrations that ranged from 0.85 $\mu\text{g/mL}$ to 1.5 $\mu\text{g/mL}$; mean concentration was 1.22 $\mu\text{g/mL}$. (Figure 8) Analysis of residuals from mixed models analysis based on statistical diagnostic tests and QQ plots revealed that residuals were normally distributed, thus fulfilling one of the basic assumptions underlying analysis of variance. In the 2^2 factorial analysis there was no significant ($P > 0.22$) interaction between storage method (serum or plasma-EDTA) and storage temperature (-20°C or -80°C) for all time points (data not shown). Furthermore, the effect of storage method was also not significant in both the 2^2 factorial ($P > 0.41$) and the single factor analysis ($P > 0.30$) involving all three storage methods at -80°C . The only significant effect on serum adiponectin concentration was storage temperature at the first three time points. (Figure 9)

SDS-PAGE and Western Immunoblotting of Canine Serum- Adiponectin protein complexes in serum obtained from clinically normal dogs were investigated via SDS-PAGE and Western Immunoblotting. Untreated samples yielded a protein band at 180 kD (LMW). In denatured serum, bands at 180 kD, 90 kD and 60 kD (LMW, trimer, and

dimer, respectively) were detected. Reduction of serum resulted in detection of two protein bands located at approximately 90 and 60 kD (trimer and dimer, respectively). Reduction and denaturation combined resulted in detection of protein bands at approximately 60 kD, 30 kD, and 28 kD (dimer, monomer, globular head region). (Figure 10)

Discussion

Circulating adiponectin complexes account for approximately 0.01% of total plasma proteins in mammalian species; plasma adiponectin concentrations range from 5-30 µg/mL in humans, 9-17.4 µg/mL in mice, and 3-12 µg/mL in arctic blue foxes (60; 129; 140). Compared with findings in those species, results of radioimmunoassay of serum samples in the present study indicated that circulating adiponectin concentrations were lower in dogs, ranging from 0.85 µg/mL to 1.5 µg/mL (mean concentration of 1.22 µg/mL). These concentrations are decreased compared with values in dogs reported by Ishioka *et al.*; those investigators used a mouse-rat adiponectin ELISA kit, which may account for the differences. Storage method (serum, plasma-EDTA, plasma-heparin) did not influence the adiponectin concentration but storage temperature did. However, the effect of temperature was not consistent. In 2 of 3 instances in which temperature effects were significant, adiponectin concentrations were higher when samples were stored at -20°C compared with storage at -80° C.

Currently, radioimmunoassay only allows for the measurement of total adiponectin and does not account for differences in the protein complexes of adiponectin present in the circulation. Therefore it is necessary to identify protein complex profiles through other methods. Via SDS-PAGE and Western immunoblotting in the present

study, canine serum yielded a protein band at 180 kD, which was analogous to the low molecular weight form (LMW) described by Pajvani *et al* (137). Denaturation yielded protein bands at 180 kD, 90 kD, and 60 kD, which represented the LMW, trimer, and dimer, respectively. Under reducing conditions, two protein bands located at 60 kD and 30 kD, (the dimeric and monomeric forms of adiponectin) were detected. Reduction and denaturation in combination resulted in protein bands at 60 kD, 30 kD, and 28 kD, these represented the dimer, the monomer, and the globular head region, respectively. The high molecular weight complex (HMW) could not be resolved under the electrophoresis conditions applied in the present study. However, preliminary data from velocity sedimentation studies performed in our lab have suggested that the HMW complex is present in canine serum.

The results of the present study seem to correlate with data obtained from humans. In human serum, adiponectin circulates as a trimer, a low molecular weight (LMW) form, and a high molecular weight complex (HMW); the LMW and HMW complexes predominate, and the smaller trimeric complex circulates at virtually undetectable concentrations (136; 156). Under reducing and denaturing conditions, multimer species of adiponectin are separated into cross-linked products whose molecular sizes are multiples of 30 kD (147).

The importance of companion animals in comparative medicine has increased with the completion of the canine gene sequence, which has a higher degree of homology with the human counterpart than sequences of the frequently studied mouse or rat (144). Diseases affecting humans and dogs are often very similar clinically, indicating that the use of dogs may be more effective than the extensive use of mice for gaining insights into

metabolic disorders of humans (39). For example, the lipoprotein profile of dogs is quite different from that of humans except when insulin resistance is induced. Normally, canine plasma is rich in high density lipoproteins (the concentration is 3 times as great as that of humans (36)), whereas human plasma is rich in low density lipoproteins (LDL) and very low density lipoproteins (VLDL) (119). However, when insulin resistance is induced through feeding a high energy diet in dogs, the lipoprotein profile is altered, resulting in increased plasma nonesterified fatty acids (NEFA) concentration and triglyceridemia (through increases in VLDL and HDL) and a decreased amounts of high density lipoprotein-total cholesterol (HDL-TC), which are the main profile changes identified in insulin resistant humans (3). Because of the similarities of the disease states in dogs and humans, these data suggest that an insulin-resistant, obese dog model could be useful in studying insulin resistance-associated dyslipidemia in humans.

In humans, the antiatherogenic effects of adiponectin have been investigated and results indicate that serum adiponectin concentrations are negatively correlated with serum triglyceride and LDL concentrations and positively correlated with serum HDL concentrations. Unlike humans, atherosclerosis is uncommon in dogs, and dogs with atherosclerosis are more likely to also have other illnesses such as diabetes mellitus and hypothyroidism, compared with dogs without atherosclerosis (71). With increasing evidence that adiponectin may physiologically regulate energy metabolism and is important in the relationship between obesity and the development of insulin resistance, adiponectin could potentially serve as a treatment target or marker for obesity and insulin resistance or as an indicator of the development of diabetes mellitus in multiple species. Further investigation into the metabolic functions of adiponectin may further elucidate its

role in the underlying pathogenesis of obesity-associated diseases dogs, as well as offer insights into the differences between metabolic diseases in humans and dogs.

Footnotes

^a Affinity Bioreagents, Golden, CO

^b LI-COR Biosciences, Lincoln, NB

^c LINCO Research, St. Louis, MO

^d Ambion, Inc., Austin, TX

^e Invitrogen, Carlsbad, CA

^f Bio-Rad, Hercules, CA

^g Bio-Rad, Hercules, CA

^h Bio-Rad, Hercules, CA

	18	32
Mouse	MLLLQALLFLLILPSHAEDDVTTEELAPALVPPP	KGTCAGWMAGIPGHP
Rat	MLLLQALLFLLILPSHEG-----ITATEGPG--	ALVPPPKETCAGWMAGIPGYP
Dog	MLLLRAVLLLLLVLPAHGQD-----SVAEGPG--	VLLPLPKGACPGWMAGIPGHP
Human	MLLLGAVLLLLLALPGHDQE-----TTTQGGPG--	VLLPLPKGACTGWMAGIPGHP
Mouse	GHNGTPGRDGRDGTTPGEKGEKGDAGLLGPKGET	GDVGMTGAEGPRGFPPT
Rat	GHNGIPGRDGRDGTTPGEKGEKGDAGVLGPKGDP	GDAGMTGAEGPRGFPPT
Dog	GHNGTPGRDGRDGTTPGEKGEKGDPLVGPKGDT	TGETGVTGVEGPRGFPPT
Human	GHNGAPGRDGRDGTTPGEKGEKGDPLIGPKGDI	GETGVPGAEGPRGFPPT
Mouse	PGRKGEPGEAAYMYRSAFSVGL	ETRVTVPNVPIRFTKIFYNQQNHYDGST
Rat	PGRKGEPGEAAYMYHSAFSVGL	ETRVTVPNVPIRFTKIFYNQQNHYDGST
Dog	PGRKGEPGESAYVHRSAFSVGL	ESRITVPNVPIRFTKIFYNLQNHYDGTT
Human	QGRKGEPGEGAYVYRSAFSVGL	ETYVTIPNMPIRFTKIFYNQQNHYDGST
	187	200
Mouse	GKFYCNIPGLYYFSYHITVYMKDVKVS	LFKKDKAVLFTYDQYQEKNVDDQA
Rat	GKFHCNIPGLYYFSYHITVYMKDVKVS	LFKKDKAVLFTYDQYQEKNVDDQA
Dog	GKFHCNIPGLYYFSYHITVYMKDVKVS	LYKKDKAVLFTYDQYQEKNVDDQA
Human	GKFHCNIPGLYYFAYHITVYMKDVKVS	LFKKDKAVLFTYDQYQEKNVDDQA
Mouse	SGSVLLHLEVGDQVWLQVYGDGDHNGLY	ADNVNDSTFTGFLLYHDTN
Rat	SGSMLLHLEVGDQVWLQVYGEEDNNGLY	ADNVNDSTFTGFLLYHDTN
Dog	SGSVLLHLEVGDQVWLQVYGDGDSYGI	YADNVNDSTFTGFLLYHDTN
Human	SGSVLLHLEVGDQVWLQVYGEGERNGLY	ADNDNDSTFTGFLLYHDTN

Figure 4: Comparison of multiple alignment sequences adiponectin in mouse, rat, dog, and human. Peptide sequences of these species have marked homology (mouse:dog, 85%; rat:dog, 83%; and human:dog, 87%). The polyclonal murine antiadiponectin antibody used for Western immunoblotting recognizes peptides 18-32 and 187-200 (highlighted region).

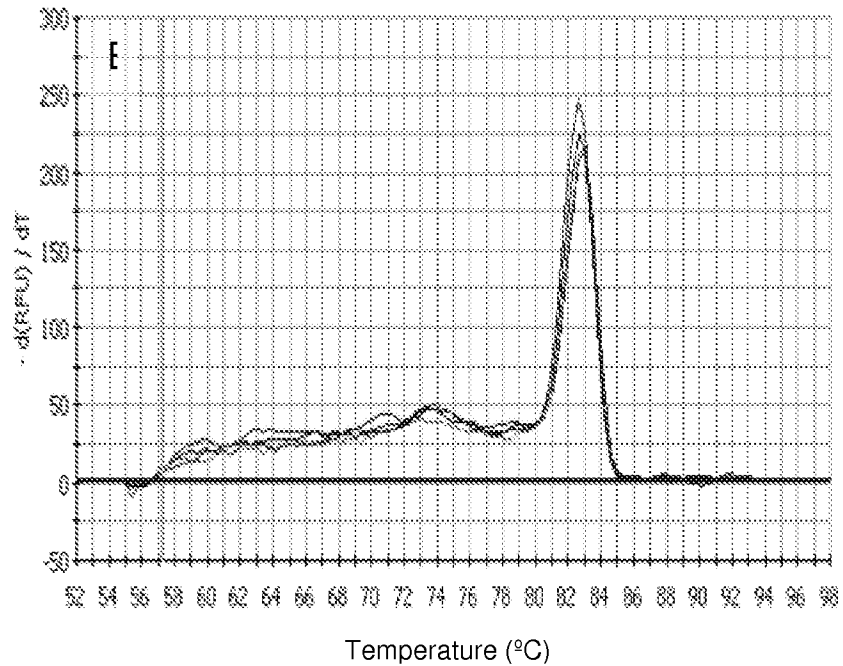
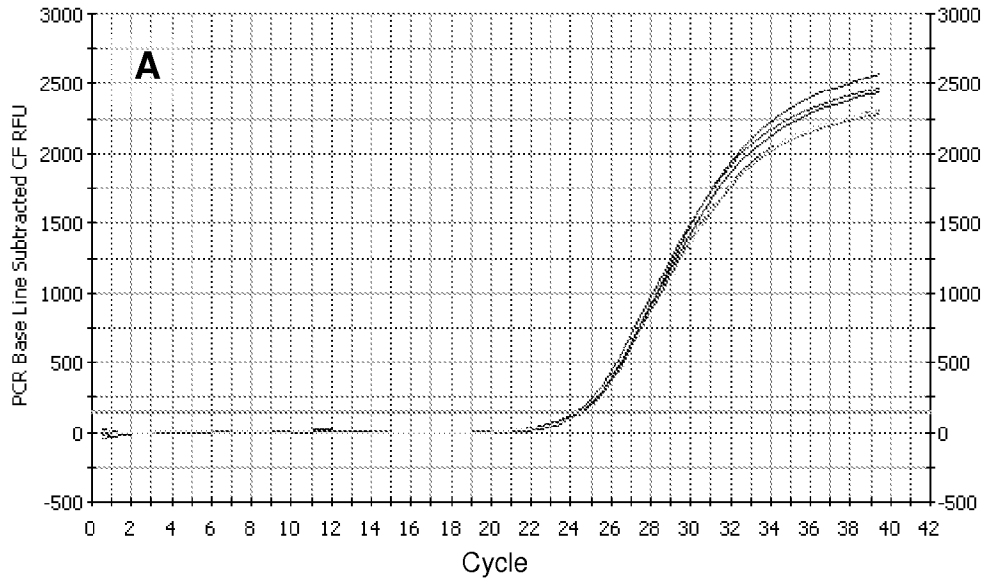


Figure 5: Assessment of adiponectin gene expression in adipose tissue obtained from the falciform ligament of a healthy dog that was undergoing routine, elective ovariohysterectomy. The visceral adipose tissue was analyzed by real-time PCR. (A.) PCR quantification plot of a single sample of canine adipose tissue run in quadruplicate. (B.) Melt curve of the PCR product from panel A demonstrating formation of a single product. CF RFU = Curve fit relative fluorescence units. $-d(\text{RFU})/dT$ = negative first derivative of the temperature versus fluorescence plotted against temperature.

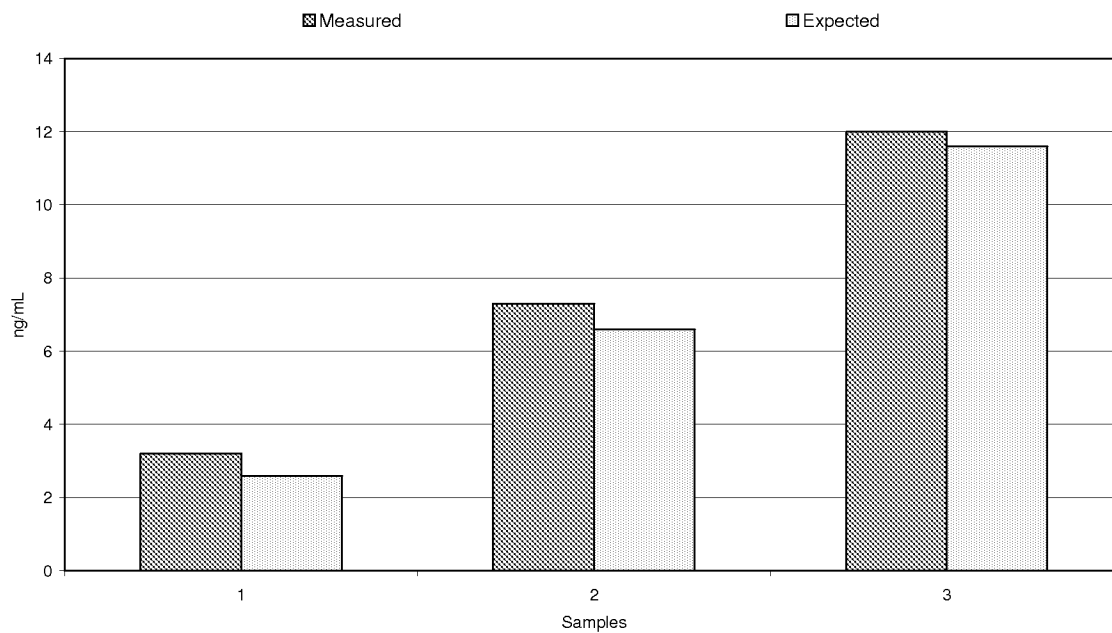


Figure 6: Canine serum samples spiked with murine adiponectin are found to exhibit additivity. Serum samples were spiked with 0.1 ng (Sample 1), 0.5 ng (Sample 2), and 1 ng (Sample 3) of murine adiponectin. Percent difference between expected and measured recovery was 24%, 11%, and 4%, respectively.

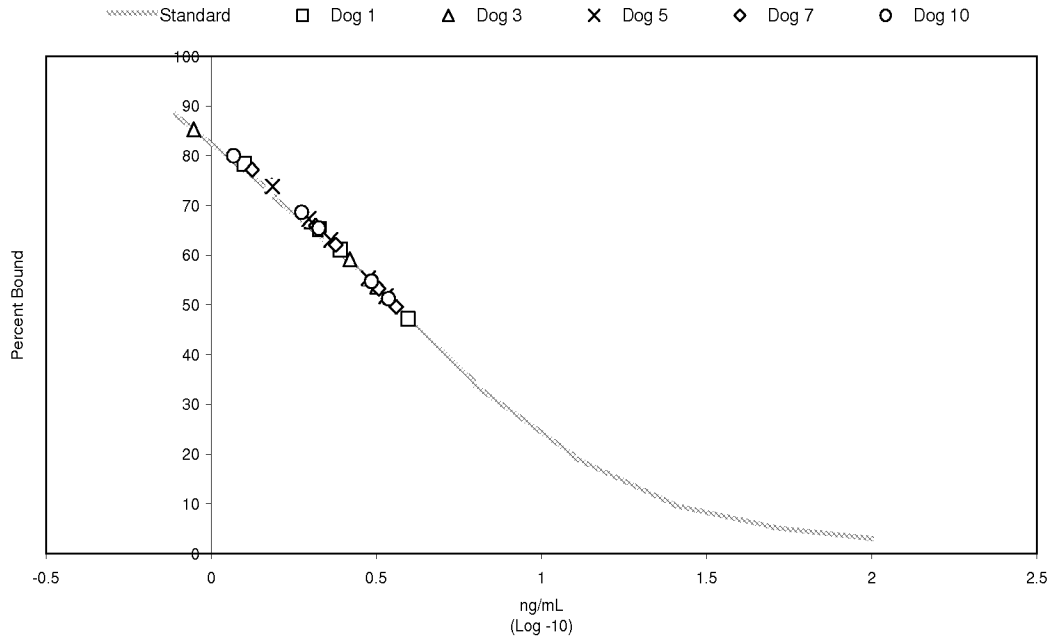


Figure 7: Evidence of parallelism of adiponectin concentrations in canine serum samples, compared with a generated standard curve. Serum samples were serially diluted (1:50, 1:100, 1:500, 1:1000, 1:5000) and adiponectin concentrations measured by RIA.

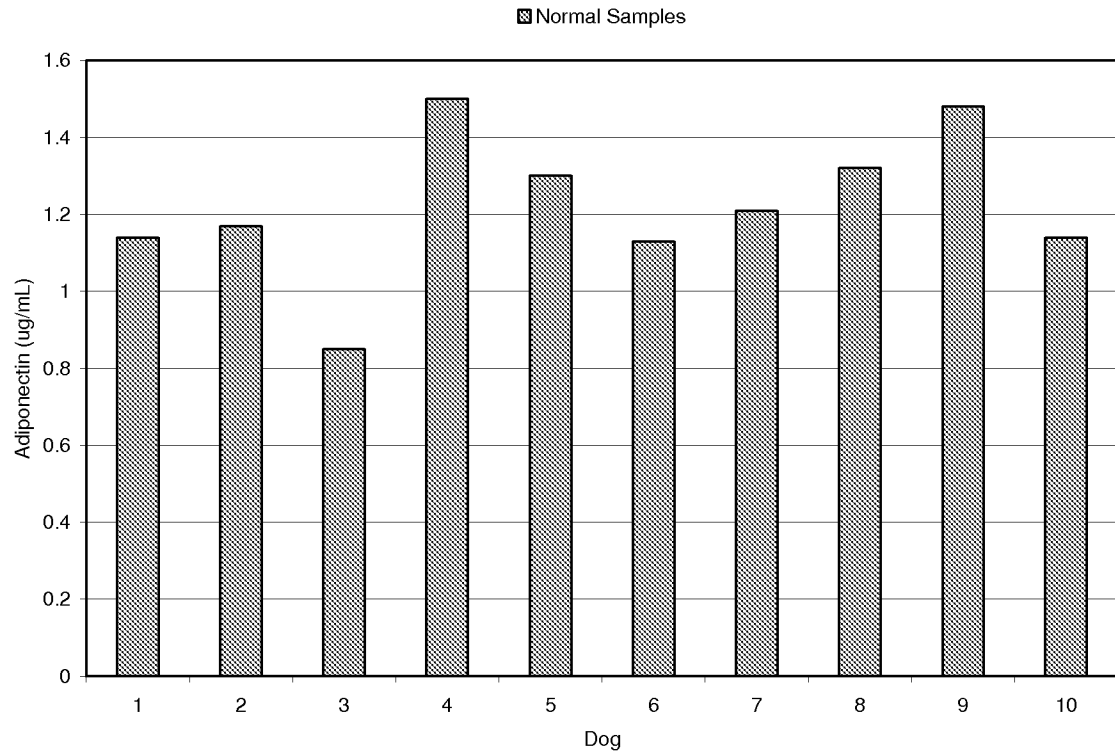


Figure 8: Serum from 10 normal dogs have serum adiponectin concentrations ranging from 0.85 to 1.5 $\mu\text{g}/\text{mL}$ with a mean concentration of 1.22 $\mu\text{g}/\text{mL}$. Serum adiponectin concentrations were measured by use of a mouse adiponectin RIA. The inter- and intra-assay coefficients were 9.7% and 9.5%, respectively.

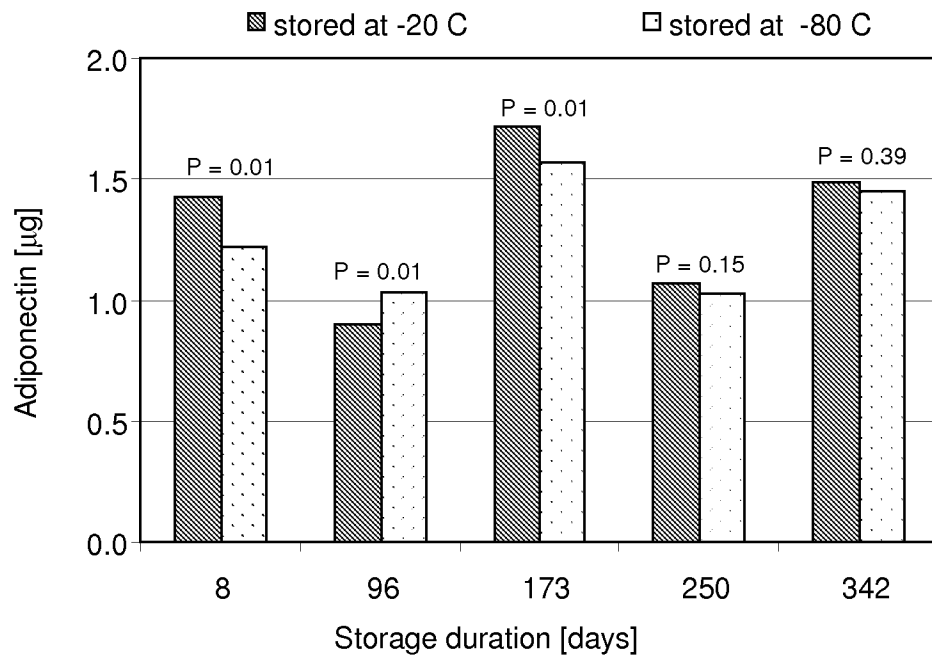


Figure 9: Effect of storage temperature on adiponectin concentrations in serum samples obtained from 10 healthy dogs after samples had been stored for as long as 342 days at -20° C (black bars) or -80° C (white bars). The p values above the bars represent the probabilities from the linear contrast of concentration at -20° C versus concentration at -80° C at each time point.

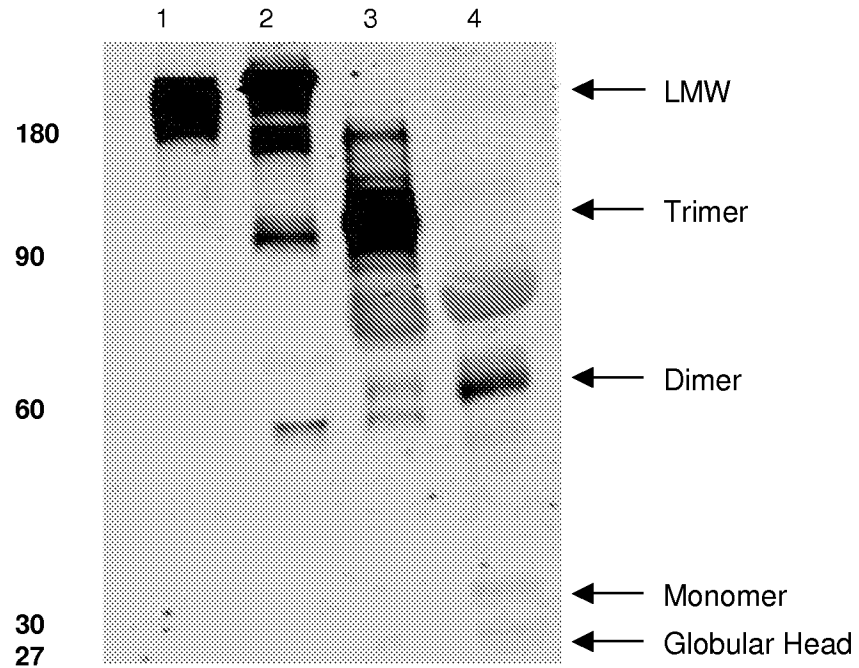


Figure 10: Results of SDS-PAGE and western blotting to evaluate adiponectin protein complexes in serum from a healthy dog after samples were exposed to various reducing and denaturing conditions. Lane 1 represents serum exposed to non-denaturing, non-reducing conditions; a 180 kD (LMW) band is visible. Lane 2 represents serum exposed to denaturing, non-reducing conditions; 180 kD, 90 kD, and 60 kD (LMW, trimer, dimer) bands are visible. Lane 3 represents serum exposed to non-denaturing, reducing conditions; 90 kD and 60 kD (trimer, dimer) bands are visible. Lane 4 represents denaturing and reducing conditions; 60 kD, 30 kD, and 28 kD (dimer, monomer, globular head) bands are visible. Resultant adiponectin protein complexes are indicated to the right of the image.

CHAPTER IV.

Adiponectin Gene Expression and Secretion in a High-Fat Fed Dog Model

Abstract

Obesity has reached epidemic proportions among adults and adolescents in the United States. Obesity is associated with a number of chronic disease conditions including hypertension, insulin resistance, type 2 diabetes, cardiovascular disease, and dyslipidemia. Adiponectin has recently emerged as a biomarker of obesity and the metabolic syndrome. Due to similarities and homology between the human and dog genome, dogs are becoming more utilized as research models of human diseases.

We examined fourteen dogs over the course of approximately 36 weeks to determine the effects of high-fat feeding and an acute increase and decrease in adiposity on adiponectin gene expression and protein secretion. After 16 weeks of feeding a high-fat diet, there was deposition of significant adipose depots with minimal changes in body weight. At the peak of adiposity, high-fat fed dogs exhibited high/normal fasting insulin concentrations with normal fasting blood glucose concentrations. There was no change in adiponectin gene expression or protein secretion (total and HMW adiponectin) in high-fat fed versus control dogs at any timepoint during the study. The results of this investigation provide evidence that an acute increase in adiposity without changes in body weight does not affect insulin sensitivity or adiponectin expression and secretion in high-fat fed dogs.

Introduction

Obesity is a major public health problem that has reached epidemic proportions in the United States and throughout the world. The prevalence of obesity among U.S. adults increased from 15% to 33% from 1976 to 2004 (70). Furthermore, approximately one-third of U.S. children are now considered overweight or obese (69). Obesity is associated with a number of metabolic and cardiovascular disease (CVD) risk factors collectively referred to as the metabolic syndrome (168). Adiponectin has emerged as a novel biomarker of the metabolic syndrome due to the association between decreased adiponectin concentrations, insulin resistance, hypertension, and dyslipidemia (124). While the mechanisms responsible for the association between hypoadiponectinemia and these conditions have yet to be fully elucidated, adiponectin has received much attention as a biomarker for the prediction of the metabolic syndrome.

Adiponectin is one of the most abundant adipokines secreted by adipose tissue. Adipokines contribute to the regulation of a variety of processes ranging from appetite and insulin sensitivity to inflammation and atherogenesis. Adiponectin, which was discovered in the late 1990s by four independent laboratories, circulates in human serum mainly as a 180-kD low molecular weight (LMW) hexamer and a high molecular weight (HMW) multimer of approximately 360 kD (106). The HMW multimer is believed to be the more biologically active form and has been shown to promote the survival of endothelial cells by preventing apoptosis (103). Adiponectin was recently determined to circulate within canine serum as multiple protein complexes (15) and is found at

concentrations ranging from 2.6 to 73.5 $\mu\text{g/mL}$, being comparable to reported concentrations in other species (83).

Although the mechanisms by which obesity increases the risk of type 2 diabetes and CVD have been studied extensively, the exact mechanisms remain unresolved. Rodents and other laboratory animal models have provided valuable insight into the underlying pathogenesis of these conditions; however, species and metabolic differences limit their inference to humans. The recently completed canine genome has been identified to have a higher degree of homology to the human genome than that which exists between the human and mouse genome (102). Diseases affecting humans and dogs are often very similar clinically, indicating that dogs may be more effective as research models than rodents for gaining insights into human metabolic disorders (39). Additional advantages to using large animal models (such as canine) include easier access to anatomic structures for detailed study (such as access to the abdominal portal vein to evaluate visceral fat signaling to the liver) and the ability to evaluate the development and/or reversal of obesity (11).

The pathological impact of obesity on metabolic diseases may be a focal area of difference between the dog and the human. Due to its anti-inflammatory, anti-atherogenic, and insulin sensitizing properties, adiponectin is a plausible candidate for studies directed towards determining the relationships and mechanistic differences of human and canine metabolic diseases attributed to obesity. The purpose of our study was to determine the influence of high-fat feeding and an acute increase and decrease of adiposity on adiponectin gene expression and secretion.

Materials and Methods

Animals

Fourteen (6 female, 8 male) sexually intact, purpose-bred hound mixes were weighed and body condition scores assessed weekly using the 9 point Nestle Purina Body Condition Score Scale (1-3 = underfed; 4-5 = ideal; 6-9 = overfed). Dogs were approximately 14, 18, and 23 months of age at timepoints 1, 2, and 3, respectively. Dogs were obtained from the Division of Laboratory Animal Health, College of Veterinary Medicine, Auburn University, and housed in indoor/outdoor kennels maintained by the Division of Laboratory Animal Health on a 12:12 light:dark cycle. All aspects of this study were approved by the Auburn University Institutional Animal Care and Use Committee prior to initiation.

Diet

All dogs were acclimated to a maintenance diet (Science Diet Adult Large Breed - 25.1% protein, 15.5% fat, 52.3% carbohydrate, and 3702 kcal/day, on a dry matter basis) and fed once daily for approximately 6 weeks prior to obtaining baseline data (Timepoint 1). Following the acclimation period, dogs were randomly divided into control (n=7) and high-fat fed (n=7) groups after separation based upon gender. The control group continued to be fed the maintenance diet once daily and the high-fat fed group received a high-fat diet designed to acutely induce adiposity [(75% of the energy allowance from a dry diet (Hill's Canine P/D) – 31.8% protein, 23% fat, 35% carbohydrate and 4511 kcal/day; and 25% from canned food (Hill's Canine P/D) – 32.5% protein, 26.6% fat, 31.6% carbohydrate, and 1415 kcal/day)] ad libitum for approximately 16 weeks

(Timepoint 2). Following data collection for timepoint 2, the high-fat fed group was returned to the maintenance diet for approximately 16 weeks in order to decrease adiposity and induce weight loss (Timepoint 3). Control dogs were fed the maintenance diet for the entire length of the study.

Intravenous Glucose Tolerance Testing (IVGTT)

After an overnight fast, dogs were catheterized in the right or left cephalic forelimb vein and given an intravenous bolus of glucose (500 mg/kg) as a 50% solution administered slowly over 30 seconds. Venous blood was collected from the cephalic catheter before dosing and at 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 minutes after dosing. Serum insulin concentrations were measured with a commercially available human radioimmunoassay (LINCO Research, St. Louis, MO). Plasma glucose concentrations were measured by the hexokinase reaction in a computerized chemistry analyzer (Hitachi 911; Roche Diagnostic Corporation, Indianapolis, IN).

Dual Energy X-ray Absorptiometry (DEXA) Scanning

After an overnight fast, dogs were anesthetized with butorphanol (0.4 mg/kg IM) and medetomidine hydrochloride (1,000 $\mu\text{g}/\text{m}^2$ of body surface area IM) (Pfizer, Inc. New York, NY) in order to provide complete relaxation and immobilization. Each dog was positioned in dorsal recumbency with hind limbs and forelimbs extended caudally. The forelimbs were placed lateral to the thoracic wall, and the spinal column was straightened dorsoventrally and craniocaudally. Each dog was secured in position by use of one inch wide medical tape that extended from the limbs, head, and across the thorax. Total body absorptiometry scans were performed on a dual energy x-ray absorptiometer (Lunar model DPX-L densitometer, Lunar Corp, Madison, WI) utilizing the fast detail

scan mode and analyzed by use of software programs designed by the manufacturer to accommodate different body weights. Data was analyzed utilizing the manufacturer's software (Lunar Pediatric software, version 1.5g, Lunar Corp, Madison, WI; Lunar Adult software, version 1.31, Lunar Corp, Madison, WI)

Surgical Biopsies

Following DEXA scans, dogs were masked with adequate isoflurane to allow for intubation and maintained on 1.5-2% isoflurane gas for surgical biopsy procedures. Approximately one gram of visceral fat was obtained by making a 0.5 cm, standard, ventral midline abdominal incision into the abdominal wall musculature and the linea alba. A small portion of the falciform ligament was exteriorized and removed utilizing sterile procedure. The linea alba and abdominal wall musculature were closed utilizing a simple interrupted pattern with absorbable suture material and the overlying skin was closed utilizing a subcuticular pattern with absorbable suture material. One gram of subcutaneous fat was obtained either from the initial incision site or from the inguinal area and closed in a similar fashion as described previously. Visceral and subcutaneous adipose tissue samples were stored in *RNAlater* (Invitrogen, Carlsbad, CA) or 10% neutral buffered formalin for subsequent analysis.

Adipocyte Morphometry

Visceral adipose tissue (from timepoints 2 and 3) fixed in 10% neutral buffered formalin was processed routinely, embedded in paraffin, and sections were stained with hematoxylin and eosin. Analysis of photomicrographs from four different fields (from each sample) was performed at 200x magnification on an IBM computer using the public domain NIH Image J software program (developed at the U.S. National Institutes of

Health and available on the Internet at <http://rsb.info.nih.gov/ij/>). Intact adipocytes were identified and counted to give the total number of adipocytes per field. The area of individual adipocytes was measured and the mean area (in micrometers) reported.

Adipokine Measurement

Canine total adiponectin was measured utilizing a canine adipocyte Lincoplex assay (LINCO Diagnostic Services, St. Charles, MO). High molecular weight (HMW) adiponectin was measured utilizing a human HMW adiponectin ELISA (LINCO Research, St. Louis, MO).

Real time PCR

Total RNA was isolated following the QIAzol/RNeasy Lipid Tissue Mini Kit protocol (QIAGEN Inc., Valencia, CA) for adipose tissue. One microgram of total RNA was converted to cDNA using the iScript cDNA Synthesis Kit (BioRad Laboratories, Inc., Hercules, CA). Gene expression was measured by real time PCR (iCycler iQ Real-Time PCR detection system, Bio Rad Laboratories, Hercules, CA). Two microliters of each RT reaction was amplified in a 30 μ L PCR containing 200 μ L of each primer and SYBR Green Super Mix (BioRad Laboratories, Hercules, CA). Samples were incubated in the iCycler for an initial denaturation at 95°C for 3.0 minutes followed by 40 PCR cycles. Each cycle consisted of 95°C for 10 seconds and 58° C for 1.0 minute.

Oligonucleotide primers used for adiponectin were TTTGGTAAAGCGAATGGG

(forward) and GGTCTTGTTGGTCCTAAG (reverse) and for GAPDH were

TGAGTATGTTGTGGAGTC (forward) and GAAGGAGCAGAGATGATG (reverse).

SYBR Green I fluorescence emission was measured after each cycle. Adiponectin mRNA concentrations were normalized to GAPDH expression. Amplification of specific

transcripts was confirmed initially by sequencing and subsequently by producing melting curve profiles during each real-time PCR run (cooling the sample to 55°C and heating to 95°C with continuous measurement of fluorescence). Gene expression data was analyzed using iCycler iQ Optical System Software (ver. 3.0a, BioRad Laboratories, Inc., Hercules, CA).

Statistical Analysis

Data are expressed as means \pm SEM. Power analyses suggested that 6 dogs would be required to meet statistical significance for total adiponectin at an effect size of 0.8 and an alpha level of 0.05. All dependant variables were analyzed using multiple repeated measures ANOVA. Insulin and glucose area under the curves for the entire 120 minute test were quantified using the trapezoidal rule (126). Graphical and formal statistical tests were used to assess normality. Pearson's product moment correlation coefficients were used to determine associations between all dependant variables. Statistical significance was accepted at $p < 0.05$. All statistical analyses were performed using the Statistical Analysis System (SAS Institute, Inc., Cary, NC).

Results

Body Composition

Body condition score was increased at timepoint 2 in high-fat fed dogs. This finding was further confirmed utilizing DEXA scanning and adipocyte morphometry. Whole body adipose tissue increased 2.6 fold in the high-fat fed dogs at timepoint 2 compared to timepoint 1 (T1 = 2260 \pm 511 grams vs. T2 = 5879 \pm 735 grams) (Table 6), with the majority of adipose tissue being measured in the trunk. Sixteen weeks of a maintenance diet significantly reduced adiposity in high-fat fed dogs at timepoint 3

compared to timepoint 2 (T2 = 5879 ± 735 grams vs. T3= 3959 ± 641 grams) (Table 6). These results were further confirmed by measurement of adipocyte area and number per field. Adipocyte area was significantly increased (HFF = 61 ± 3 micrometers vs. C = 42 ± 3 micrometers) and number per field (HFF = 116 ± 6 vs. C = 161 ± 9) decreased in HFF dogs at timepoint 2 compared to control, however, at timepoint 3, adipocyte area and number per field were not significantly changed compared to control (Figure 11). Body weight changes were not significant despite a 15.4 % increase at timepoint 2 in high-fat fed dogs.

IVGTT

Despite a 2.6 fold increase in adiposity at timepoint 2, high-fat fed dogs exhibited no significant changes in fasting insulin concentrations (Table 7). Insulin area under the curve (IAUC) was not significantly different between timepoints; however, a group effect was observed in which high-fat fed dogs had a higher IAUC compared to control dogs throughout the study period. (Figure 12)

Adiponectin

Adiponectin gene expression in visceral and subcutaneous fat (Figure 13), total adiponectin serum concentrations (T1 = 30.4 ± 3.4 µg/mL; T2 = 47.0 ± 12.8 µg/mL; T3 = 40.3 ± 5.7 µg/mL), and high molecular weight adiponectin concentrations (T1 = 14.6 ± 1.2 µg/mL; T2 = 17.4 ± 2.3 µg/mL; T3 = 16.5 ± 1.5µg/mL) remained unchanged (based on statistical evaluation) during the course of the study (Figure 14). HMW adiponectin comprises approximately 38% of total circulating adiponectin concentrations (Table 8).

Discussion

The association between obesity, insulin resistance, and other risk factors for chronic diseases in both humans and domestic animals is consistent and strong. For many years, dogs have been utilized as a model of human metabolic syndrome (13). Dogs are genetically similar to humans (~ 90%) and clinically often suffer from similar diseases, which indicates that the dog might be an appropriate model for gaining insights into numerous human metabolic disorders (12; 39).

Adiponectin was recently identified in dogs by two independent research groups (16; 83). However, it is unclear how plasma adiponectin concentrations change during the progression from a lean to obese state. Therefore, the question addressed in the present study was whether a model of acute canine adiposity (induced by feeding a commonly utilized, high-fat diet similar to the Western diet) would result in detectable changes in adiponectin gene expression and total and HMW adiponectin secretion during periods of acute increases and decreases in adiposity.

Our results demonstrate that large hound mixes fed a high-fat diet over a period of 16 weeks develop a significant degree of adiposity. However, the deposition of increased adipose tissue did not result in a significant change in body weight. Similar findings were observed by Kim *et al.* in a recent study in which male mongrel dogs were fed a high-fat diet for 12 weeks, resulting in a substantial increase in body fat without significant changes in body weight (99). In humans, weight gain precedes the development of insulin resistance. Men that have recorded weight gains of 10-19%, 20-29%, and >30% were 3, 4.7, and 10.6 times more likely to develop the metabolic

syndrome. Additionally, each 5% increase in weight gain over the baseline weight (at 20 years of age) was associated with a 38% greater risk of hyperinsulinemia by middle age (41). Visceral adipose tissue is highly correlated to the metabolic syndrome. Kelley *et al* (92) demonstrated that women and men diagnosed with the metabolic syndrome exhibited 2.6 and 2.7 fold increases in total truncal adipose tissue (similar to our studies) as measured by dual energy x-ray absorptiometry (DEXA) and a 49% increase in body weight compared to controls.

Despite the increase in total body fat in high-fat fed dogs, fasting glucose concentrations were unaffected by high-fat feeding over the course of the study as were fasting non-esterified fatty acids (NEFAs) (data not shown). Insulin concentrations in normal fasting dogs ranged from 5-20 $\mu\text{U}/\text{mL}$, with 10-20 $\mu\text{U}/\text{mL}$ considered high/normal and 5-10 $\mu\text{U}/\text{mL}$ considered low normal. High-fat fed dogs had high/normal concentrations of fasting insulin, indicative of a minimal degree of insulin resistance. These findings are similar to those reported by Kim *et al.* in their high-fat diet dog model. Compared to our model in which only 3 of 7 dogs had increased fasting plasma insulin concentrations at peak adiposity, Kim's model developed a higher degree of insulin resistance and fasting hyperinsulinemia; however, like our model, exhibits no change in fasting glucose or NEFA concentrations (98). In other studies, it has been suggested that the maintenance of normal glucose concentrations despite the development of insulin resistance indicates that increased glucose could not be the sole factor responsible for insulin resistance or compensatory hyperinsulinemia in the fat-fed dog model (97).

Despite the development of significant adipose depots in high-fat fed dogs and the presence of high/normal fasting insulin concentrations (which are assumed to eventually

lead to insulin resistance), adiponectin gene expression was unaffected as were total and HMW serum concentrations.

This is the first report identifying the presence of HMW adiponectin in the dog. In humans, the HMW complex is believed to be the more biologically active of the circulating multimers and has been shown to be decreased in metabolically obese, normal weight human subjects characterized by excess visceral fat and insulin resistance (91). The secretion of HMW adiponectin has also been shown to increase upon treatment with the insulin sensitizing agent pioglitazone in cultured primary human and mouse adipocytes (14). This knowledge has led to increased interest in examining total and HMW adiponectin and their possible metabolic and anti-atherogenic effects in the dog.

There have been few studies published examining adiponectin in the dog (17; 39; 58; 83). Through these studies, there is evidence to support that the adiponectin gene is expressed exclusively in white adipose tissue depots at high levels (39). In contrast to our findings, two groups have reported decreases in adiponectin gene expression in a 55 week weight gain study (57) and total serum protein concentrations in experimentally induced weight gain (14 weeks) and naturally occurring obese dogs (83). Each study utilized single sex, gonadectomized beagles ranging from 1-3 years in age. It is well documented that gonadectomy increases the risk of obesity in dogs, with gonadectomized dogs being twice as likely to be overweight than intact dogs (21). The fact that our study was comprised of intact males and females may have attributed to the lower degree of weight gain compared to the aforementioned studies. Additional factors that could have accounted for the minimal degree of weight gain in our dogs include the composition of the diets utilized as well as the duration of the obese state. For instance, once obesity was

achieved, Gayet *et al.* allowed study dogs to maintain this state for 8 weeks (56). In studies by Kim *et al.*, adiposity was induced through feeding of a maintenance diet supplemented with cooked bacon grease (96; 100).

Studies in both humans and rodents have shown that adiponectin gene expression and protein secretion are also affected by sex hormones. Total serum adiponectin concentrations are higher in women than men with HMW adiponectin accounting for this difference (171). Furthermore, castration of mice induces a dramatic elevation of HMW adiponectin with no effect on LMW or trimeric adiponectin. In contrast, ovariectomy results in a decrease in adiponectin mRNA with no change in total adiponectin protein concentrations (64). It still remains that the interplay of sex hormones in the development of obesity and adiponectin gene expression/protein secretion in dogs needs to be further studied in order to better understand the significance of adiponectin in this model.

In conclusion, we demonstrate that adiponectin gene expression and protein secretion (total and HMW) are unaffected in intact, nutritionally obese dogs with normal/high fasting insulin concentrations induced by high-fat feeding. Our study is the first to measure the HMW adiponectin protein complex in canine serum. Additionally, this study was designed to evaluate the effect of decreased adiposity (at timepoint 3) on metabolic parameters and adiponectin. Adiposity did not return to baseline at timepoint 3 and there were no significant changes in metabolic parameters or adiponectin. It has been suggested that insulin resistance that develops with acute canine obesity does not appear to involve changes in adipokines, although it is certainly likely that adipokines are important over the longer term (10). With this in mind, adiponectin could still prove to be a useful clinical marker to determine the duration of obesity in companion animals as

well as to assess the severity of tissue injury due to metabolic perturbations. More studies are necessary to further elucidate the role of adiponectin in human and canine obesity.

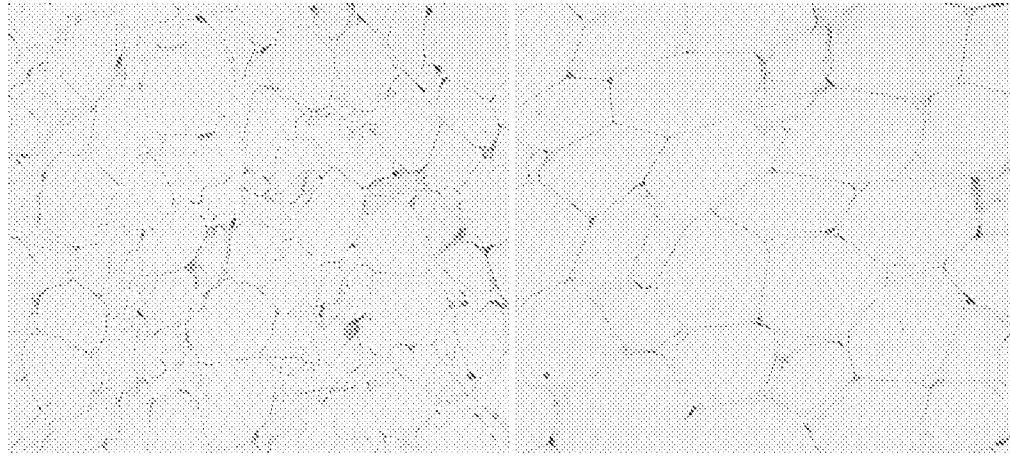
Acknowledgements

The authors would like to thank the Division of Laboratory Animal Health and their staff for assistance with animal care and handling; Drs. Stephen Lenz, William Brawner, Michael Tillson, and Mrs. Betty Files for their assistance with animal surgeries and DEXA scanning; Jacqueline Nobles, Emily Champion, Leah Ann Kuhnt, Katie Colbert, and Victoria Light for their assistance with IVGTT; Dr. Curt Bird, Patricia DeInnocentes, Dr. Elaine Coleman, Dr. Mark Carpenter, and Colin Rogers for their assistance during the course of this study.

Table 6: Body composition parameters for control and high-fat fed dogs at timepoints 1, 2, and 3.

	T1	T2	T3
Body Weight (kg)			
Control	23.8 ± 1.0	23.6 ± 1.3	30.0 ± 1.6
High-Fat Fed	21.8 ± 0.7	25.1 ± 0.7	23.2 ± 0.7
Total Body Fat (grams)			
Control	2403 ± 561	2642 ± 448	4547 ± 884
High-Fat Fed	2260 ± 511 ^a	5879 ± 735 ^b	3959 ± 641 ^a
Total Lean Tissue (grams)			
Control	18248 ± 694	19076 ± 831	19760 ± 981
High-Fat Fed	16687 ± 653	17428 ± 994	17392 ± 826
Body Condition Score			
Control	6.0 ± 0.3	5.1 ± 0.1	5.3 ± 0.2
High-Fat Fed	5.6 ± 0.3 ^a	6.7 ± 0.3 ^b	5.7 ± 0.4 ^a

Results are expressed as means ± SEM. Variables with the same letter are similar. $p < 0.05$ for all.



	Control		HFF	
	Area	Number	Area	Number
T2	42 ± 3	161 ± 9	61 ± 3*	116 ± 6*
T3	48 ± 6	134 ± 13	49 ± 3	135 ± 4

Figure 11: Morphometric analysis of canine visceral adipose tissue at T2 and T3. Photomicrographs are representative fields of visceral adipose tissue in control and high-fat fed dogs at T2 taken at 200x magnification. Adipocyte area in high-fat fed versus control dogs was significantly increased at T2. Number of adipocytes per field was significantly decreased in high-fat fed versus control dogs at T2. No significant differences in adipocyte area or number were identified between high-fat fed and control dogs at T3. Adipocyte area is measured in micrometers; number per field is measured as absolute number. Data are expressed as means ± SEM. P < 0.05.

Table 7: Results of IVGTT experiments in control and high-fat fed dogs at timepoints 1, 2 and 3.

	T1	T2	T3
Fasting Insulin ($\mu\text{U}/\text{mL}$)			
Control	13.6 \pm 4.9	7.6 \pm 0.8	7.5 \pm 1.0
High-Fat Fed	8.0 \pm 1.5	11.2 \pm 1.7	11 \pm 1.6
Fasting Glucose (mg/dL)			
Control	77.1 \pm 2.2	85.4 \pm 1.8	77.9 \pm 3.7
High-Fat Fed	76.0 \pm 2.8	82.4 \pm 2.9	84.0 \pm 2.7
Insulin:Glucose Ratio ($\mu\text{U}/\text{mL}:\text{mg}/\text{dL}$)			
Control	0.17 \pm 0.06	0.09 \pm 0.01	0.1 \pm 0.01
High-Fat Fed	0.10 \pm 0.02	0.14 \pm 0.02	0.13 \pm 0.02
Insulin AUC ($\mu\text{U}/\text{mL} \times 120 \text{ min}$)			
Control	1539 \pm 159	1614 \pm 218	2032 \pm 326
High-Fat Fed	1947 \pm 298	2443 \pm 133	2292 \pm 227
Glucose AUC (mg/dL \times 120 min)			
Control	15823 \pm 1631	13656 \pm 650	13609 \pm 978
High-Fat Fed	15358 \pm 867	15283 \pm 780	13072 \pm 525

Data are expressed as means \pm SEM.

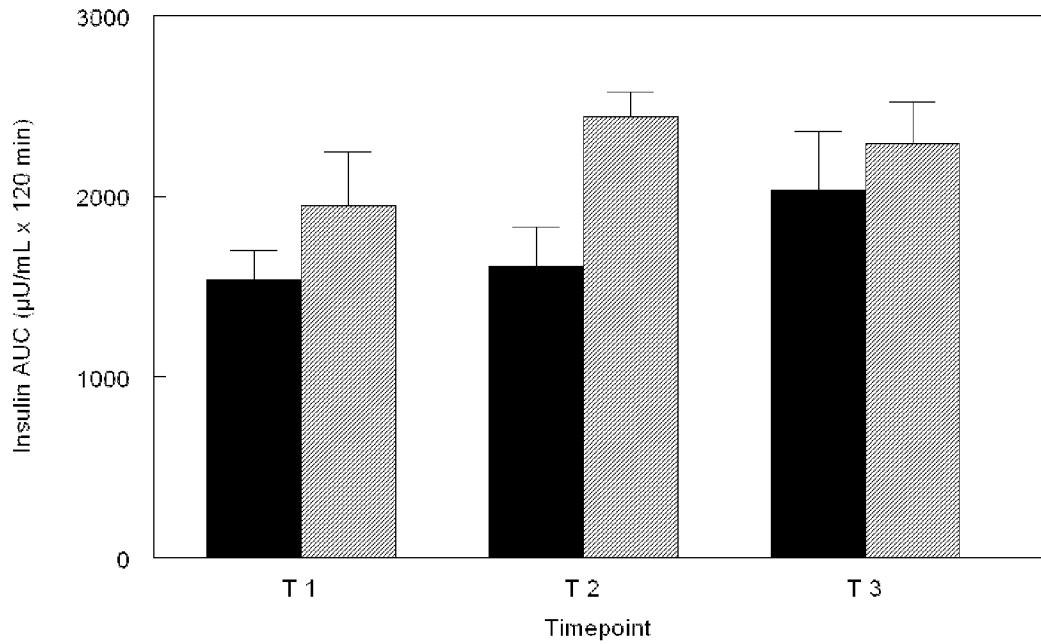


Figure 12: Insulin area under the curve (IAUC) in control fed (solid black) and high-fat fed (patterned black) dogs at all three timepoints. No significant differences were observed between control and high-fat fed dogs at each timepoint; however, high-fat fed dogs consistently had higher IAUCs than control dogs during the course of the study. $P < 0.05$

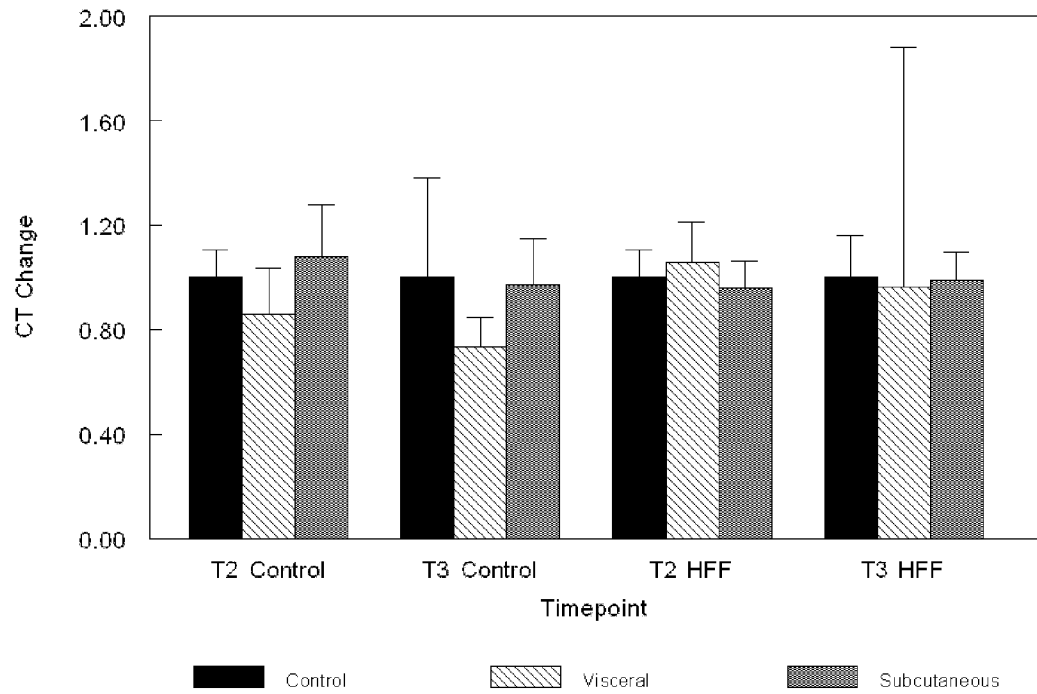


Figure 13: Adiponectin gene expression in visceral and subcutaneous adipose depots is unaffected in both high-fat fed and control dogs at timepoints 2 and 3.

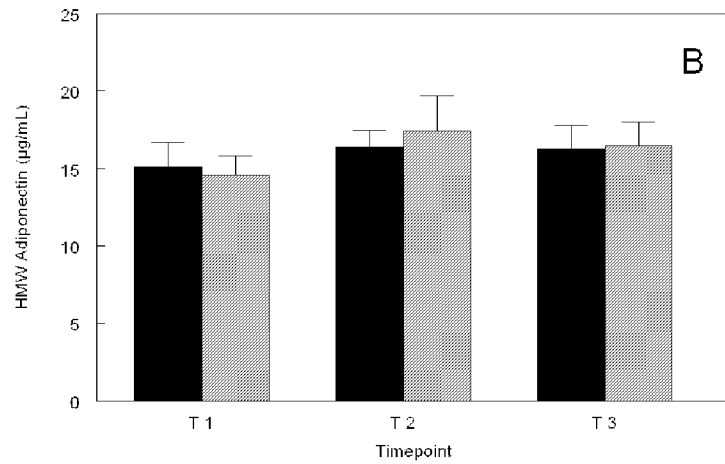
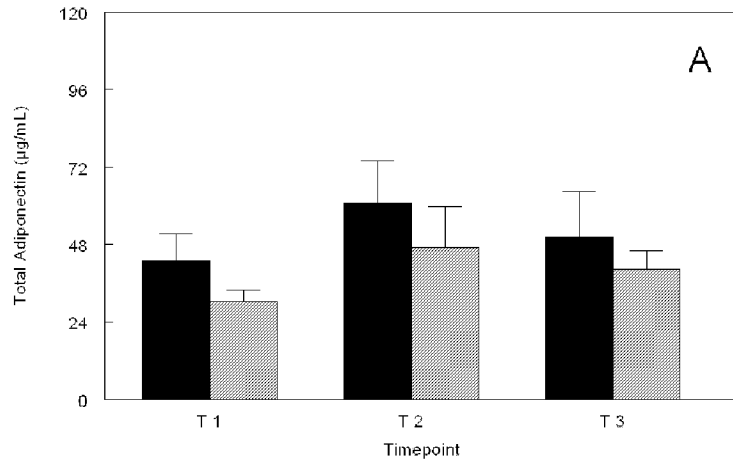


Figure 14: Total (A) and high molecular weight (B) adiponectin serum concentrations. Total and HMW adiponectin concentrations were unaffected in control fed (solid black) and high-fat fed (patterned black) dogs at all three timepoints.

Table 8: Serum adiponectin concentrations at timepoints 1, 2 and 3.

	T1	T2	T3
Total Adiponectin (µg/mL)			
Control	43.0 ± 8.3	60.8 ± 13.1	50.2 ± 14.2
High-Fat Fed	30.4 ± 3.4	47.0 ± 12.8	40.3 ± 5.7
HMW Adiponectin (µg/mL)			
Control	15.1 ± 1.6	16.4 ± 1.1	16.3 ± 1.5
High-Fat Fed	14.6 ± 1.2	17.4 ± 2.3	16.5 ± 1.5

Data are expressed as means ± SEM.

CHAPTER V.

CONCLUSIONS

Obesity has long been recognized in humans as a major public health problem. It has reached epidemic proportions among adults and adolescents in the United States. Obesity in humans is associated with a number of chronic disease conditions including hypertension, insulin resistance, type 2 diabetes, dyslipidemia, and cardiovascular disease. Paralleling human trends, obesity has become the most common nutritional disorder reported in veterinary medicine for companion animals. Recently, the importance of companion animals in comparative medicine has been realized with finalization of the dog genome. In addition to possessing 90% homology to the human genome, dogs develop similar clinical syndromes as those observed in humans, increasingly making them more popular as research models for human diseases. In addition to shortening the lifespan of dogs, obesity is also known to exacerbate numerous metabolic related syndromes, including the development of insulin resistance.

In contrast to humans, the dog is substantially resistant to the development of atherosclerosis and hypercholesterolemia. Since the adipocyte derived protein adiponectin not only exhibits decreased serum concentrations in obesity, but also possesses protective actions against the initiation and progression of atherosclerosis, we chose to study this protein in the canine.

We initially identified and characterized adiponectin in the canine. Adiponectin gene expression was demonstrated in white adipose tissue harvested from the falciiform ligament of a clinically normal dog. Additionally, multiple protein complexes, including the low molecular weight complex (180 kD), the trimer (90 kD), the dimer (60 kD), and the monomer (30 kD) were identified utilizing SDS-PAGE and immunoblotting with reduction and denaturation techniques. The high molecular weight complex could not be identified utilizing these techniques; however, it was identified later by using a human HMW ELISA. Total circulating adiponectin concentrations were characterized by use of a murine adiponectin radioimmunoassay and were found to range from 0.85 – 1.5 $\mu\text{g/mL}$, which is an extremely low range when compared to other species. Experiments utilizing recently developed canine specific assays have revealed the adiponectin range in dogs to be 2.6 to 73.5 $\mu\text{g/mL}$ (83).

After determining that canine adiponectin circulated as similar protein complexes as those reported in the human and rodent literature, studies were conducted to determine how the induction of adiposity in a high-fat fed dog model would affect adiponectin gene expression and protein secretion. After a 16-week feeding period, dogs on a high-fat diet had significantly higher body condition scores and developed a significant degree of adiposity with minimal changes in weight gain. These dogs also exhibited high/normal concentrations of fasting insulin, indicating a minimal degree of insulin resistance. Despite changes in body composition and metabolic parameters, adiponectin gene expression and secretion of both total and high molecular weight adiponectin were found to be unaffected.

Data from these studies provides evidence that adiponectin circulates in canine serum as similar protein complexes as those previously reported in human and rodent literature. Additionally, nutritionally obese, normal weight dogs with minimal development of insulin resistance show no evidence of changes in adiponectin gene expression or secretion of total or high molecular weight adiponectin. These findings are particularly interesting, as they are different to what is known about adiponectin in humans. Acute deposition of adipose tissue in humans is associated with a rapid decrease in adiponectin serum concentrations. In the dog, however, acute adiposity was not associated with changes in adiponectin gene expression or protein secretion. In humans, it also has been shown that acute adiposity induces macrophage infiltration into the adipose interstitium (30). Macrophages are known to promote insulin resistance through the production of pro-inflammatory cytokines such as IL-6 and TNF α (42). Infiltration of macrophages into adipose tissue has yet to be studied in the dog; however, preliminary studies in our laboratory indicate that acute adiposity does not induce macrophage infiltration in an acute canine model of adiposity.

Future studies should be directed towards comparing adiponectin in the dog versus humans and the possible differences which exist between the species both metabolically and immunologically. The dog is already commonly utilized as a model of human metabolic syndrome. Uncovering the mechanisms responsible for the association between decreased adiponectin serum concentrations and the metabolic and cardiovascular risk factors which collectively comprise this syndrome could result in beneficial measures for both the prevention and treatment of chronic obesity-related disease conditions in both humans and dogs.

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