EFFECTS OF ENHANCED MUSCLE GROWTH BY MYOSTATIN PROPEPTIDE TRANGENE AND DIETARY FAT CONTENT ON GENE EXPRESSION OF ADIPONECTIN, ADIPONECTIN RECEPTORS, PPAR-α, AND PPAR-γ

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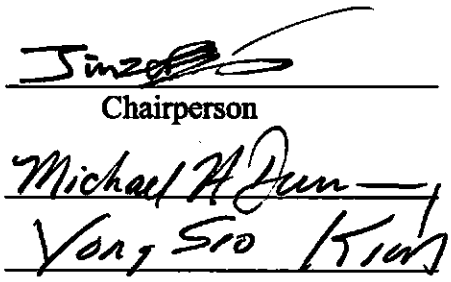

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ABSTRACT

Myostatin, a member of the transforming growth factor-β superfamily, is a highly conserved negative regulator of muscle growth which undergoes post-translational modification to yield the active form. We have demonstrated that transgenic over-expression of myostatin propeptide dramatically enhanced skeletal muscle development and decreased fat mass. By feeding the transgenic mice either a high-fat diet or normal-fat diet we found that transgenic, high-fat diet mice had improved insulin sensitivity, normal fat deposition, enhanced muscle growth, and significantly higher levels of circulating adiponectin compared to wild-type mice. Adiponectin is known to ameliorate insulin resistance and increase fatty acid oxidation. We theorized that the interaction between high-fat diet and myostatin propeptide would increase adiponectin mRNA expression in fat tissue depots and corresponding adiponectin receptor mRNA expression in muscle and liver. Results from real-time PCR analysis indicated transgenic mice fed a high-fat diet displayed increased adiponectin mRNA expression in epididymal fat by 2-fold over wild-type littermates. These mice also displayed increased expression of PPAR-α and PPAR-γ above the other three groups in epididymal fat. The wild-type mice fed a normal-fat diet expressed the most AdipoR1 and R2 in liver tissue, 1.03-fold and 1.32-fold over the other groups, respectively. The transgenic mice fed a high-fat diet did not show increased mRNA level of either receptor in muscle or liver tissue. The increase in expression of adiponectin mRNA may partially explain why the high-fat diet did not cause obesity and insulin resistance in transgenic mice.

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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

The prevalence of adult obesity is steadily increasing throughout the country as reported by the Behavioral Risk Factor Surveillance System (BRFSS) and the Centers for Disease Control (CDC). The most recent data indicates that among the American population surveyed 60.5% were overweight, 23.9% were obese, and 3% were extremely obese (Blanck et al. 2006). In 2005, there were three states (Louisiana, Mississippi, and West Virginia) with an obesity prevalence over 30% (Blanck et al. 2006). Many different factors affect the development of obesity and, therefore, it is thought to be a polygenic disorder associated with excess caloric intake along with a sedentary lifestyle. Genetic differences may play a role in the location and the extent to which adipose tissue accumulates and how energy is utilized, but ultimately energy which is not expended will be stored. Energy balance is the key to maintaining a healthy body weight. When less energy is expended than consumed, the excess energy will be stored in adipose tissues. Obesity is a risk factor for development of several different chronic diseases such as type 2 diabetes, cardiovascular disease, and some types of cancer (Kissebah et al. 2000; Grundy et al. 2004). As Americans are becoming more overweight and obese, the prevalence of type 2 diabetes and other chronic diseases are increasing. Diabetes increases risk for coronary heart disease, stroke, and hypertension. The health complications of diabetes are potentially serious and include nephropathy, neuropathy, amputation of limbs, and blindness (Anonymous 2005). A precursor to diabetes is insulin resistance which is defined as a weaker-than-expected response to insulin which

leads to hyperglycemia and excess insulin secretion by pancreatic cells. This state is termed pre-diabetes (Gil-Campos et al. 2004). Factors produced by adipose tissue may play a large role in obesity and the development of insulin resistance or diabetes. The intention of this literature review is to discuss the function of adiponectin and its effects on chronic disease, the functions of nuclear transcription factors PPAR-α and PPAR-γ and their effects on adiponectin secretion, and the factors which may induce or inhibit the expression of adiponectin receptors AdipoR1 and AdipoR2.

ADIPOCYTES AND ADIPOCYTOKINES

Adipocytes - Adipocytes can vary in size from 20-200 μm (Fruhbeck et al. 2001), and their size can have an impact on their ability to respond to insulin and to secrete adipocytokines such as adiponectin (Hotta et al. 2001). One study by Okuno *et al*, 1998, involved obese Zucker rats and found that the adipocytes in obese rats with insulin resistance were bigger than those in lean rats without insulin resistance. Treatment with troglitazone, a thiazolidinedione (TZD) used to treat type 2 diabetes, increased the number of adipocytes present, but did not significantly alter the total fat mass. Investigators discovered that troglitazone induced differentiation of the adipocytes which lead to a decrease in the individual adipocyte mass. The decrease in adipocyte size was accompanied by lower levels of free fatty acids, tumor necrosis factor-α (TNF-α), and leptin. This was also accompanied by increased insulin sensitivity in the rats (Okuno et al. 1998). Small white adipocytes are able to take up and oxidize more glucose and are more sensitive to the anti-lipolytic action of insulin. This leads to lower levels of circulating free fatty acids and triglycerides, which is thought to improve insulin sensitivity. Another key finding of Okuno *et al*'s 1998 experiment was that mRNA

expression levels of TNF-α were elevated in mesenteric and retroperitoneal adipose tissues but not in subcutaneous or epididymal adipose tissues. This supports the theory that different adipose tissues display different patterns of gene expression within the same subject (Okuno et al. 1998). Other studies reinforce this theory, noting that a high-fat diet can induce adipocyte hypertrophy which induces factors such TNF-α and free fatty acids to be released that can then lead to insulin resistance and obesity (Kadowaki et al. 2003).

Visceral obesity, or accumulation of adipose tissue in the abdominal area, has been highly associated with increased risk for insulin resistance, hyperglycemia, dyslipidemia, hypertension, and type 2 diabetes. Several studies indicate that the anatomical location of the adipose tissue can affect its endocrine function. Visceral adipose tissue hormones are secreted into the hepatic portal vein which leads directly to the liver and can have a greater effect on liver function than hormones secreted by the subcutaneous fat. Visceral adipose tissue is metabolically very active and releases its free fatty acids into portal circulation. This can enhance lipid synthesis in the liver and lead to insulin resistance, hyperlipidemia, and atherosclerosis (Matsuzawa et al. 1995; Funahashi et al. 1999; Grundy et al. 2004; Kershaw et al. 2004). A study done by Surwit *et al*, 1995, demonstrated that the genotype of the C57BL/6J mouse made them more susceptible to development of severe obesity, particularly mesenteric obesity, than the control strain when on the same diet. Researchers investigated the different effects a high-sucrose or a high-fat diet would have on the mice. This experiment determined that fat intake had a greater impact on the development of obesity and diabetes than sucrose intake (Surwit et al. 1995). The high-fat diet induced hyperplasia and hypertrophy of adipocytes, particularly in the mesenteric and epididymal fat pads compared to animals

on the control or high-sucrose diets. The researchers theorized that the ability to store fat is more important in the development of obesity than the actual contribution of ingested calories from the high-fat diet (Surwit et al. 1995). A more recent study done on C57BL/6J mice found that body weights increased more in mice fed a high-fat diet than mice fed a high-sucrose diet (Sumiyoshi et al. 2006). The mice on the high-fat diet had higher plasma levels of triglycerides, cholesterol, and free fatty acids. Also, the livers of the high-fat diet mice accumulated more triglycerides, and the number and the diameter of the adipocytes also increased. The high-fat diet mice also showed an increase in visceral fat mass compared to the high-sucrose diet mice. Researchers concluded that a high-fat diet in mice produces characteristics more closely related to metabolic syndrome than a high-sucrose diet (Sumiyoshi et al. 2006).

Adipocytokines - Adipose tissue was originally believed to be a relatively inert storage depot for energy, however it is now seen as a highly active and regulated secretory organ which can affect energy homeostasis. There are many factors produced by the adipose tissue which have been found to regulate energy balance, glucose levels, and lipid levels. Adipocytes are thought to play a critical role in energy regulation and homeostasis with the factors they release being termed adipocytokines or adipokines (Fruhbeck et al. 2001; Gil-Campos et al. 2004). These factors include leptin, adiponectin, resistin, angiotensin, and others, and are credited for the endocrine function of adipose tissue (Ahima 2006). The modification in expression of the adipocytokines may lead to the development of obesity and obesity-related disorders (Boucher et al. 2005).

Since the identification of leptin, many researchers have begun to investigate many of the regulatory and signaling molecules secreted by adipose tissue (Maeda et al.

1997; Juge-Aubry et al. 2005). Leptin acts as a satiety signal which regulates appetite and weight in rodents and humans by stimulating energy expenditure and suppressing food intake. Animals and humans which lack functional leptin become extremely obese, but common forms of obesity are also characterized by high levels of circulating leptin. There has not been much indication that administration of leptin in humans would decrease food intake or increase energy expenditure to the degree which would be necessary to stimulate weight loss (Koerner et al. 2005). High levels of circulating leptin have also been associated with increased pathogenesis of cancer (Kershaw et al. 2004; Koerner et al. 2005). Researchers have found the association between obesity and cancer to be of interest and consequently have discovered that leptin acts as a mitogen which is able to enhance proliferation of esophageal, breast, and prostate cancer cell lines. (Somasundar et al. 2003) Leptin has been found to stimulate fatty acid oxidation through activation of AMP-activated protein kinase (AMPK) and increase glucose uptake by increasing GLUT-4 content on cellular membranes (Dyck et al. 2006). Zhang *et al*, 2002, demonstrated that the rates of leptin mRNA translation and protein secretion were markedly different in three distinct adipose tissues. They concluded that the differences in leptin secretion were primarily due to the differences in adipocyte volume among the fat depots (Zhang et al. 2002). Guo *et al*, 2004, also found significant differences between leptin mRNA expression and adipocyte volume between distinct fat depots. They also examined the differences in leptin expression relative to adipocyte volume. The ratio of leptin expression to adipocyte volume was significantly lower in epididymal and retroperitoneal fat compared to subcutaneous fat in obese subjects (Guo et al. 2004).

Researchers concluded that obesity has differential effects on leptin gene expression in the three fat depots described above (Guo et al. 2004).

TNF-α and interleukin-6 (IL-6) are pro-inflammatory cytokines which are involved in the body's defense mechanisms. They stimulate glucose uptake into adipocytes and inhibit lipoprotein lipase (LPL) activity (Mohamed-Ali et al. 1998). Adipose tissue increases expression of TNF-α in states of obesity and insulin resistance. TNF-α has been implicated in insulin resistance because of its ability to interfere with insulin receptor signaling, decrease fatty acid oxidation, stimulate lipolysis, and down regulate expression of GLUT-4 in fat and muscle (Kershaw et al. 2004; Kokta et al. 2004). Feeding of a high-fat diet has been reported to significantly increase expression of TNF-α mRNA in fat pads of rats (Fruhbeck et al. 2001). Serum levels of IL-6 have been reported to increase in type 2 diabetes and metabolic syndrome and decrease after weight loss. Circulating levels of IL-6 have also been shown to strongly correlate with body mass index (BMI), and there have been depot-specific differences in regards to quantitative contribution of secreted IL-6 to the serum pool. IL-6 impairs insulin signaling through decreased phosphorylation of IRS-1 and protein kinase B (PKB/Akt) and has also been shown to increase hepatic triglyceride secretion which may contribute to the hypertriglyceridemia seen in visceral obesity (Fruhbeck et al. 2001; Juge-Aubry et al. 2005).

Some of the other adipocytokines released by adipose tissue are resistin, acylation stimulating protein (ASP), plasminogen activator inhibitor-1 (PAI-1), and adiponectin. Resistin also has been associated with increased insulin resistance and reduced glucose tolerance. ASP promotes triglyceride synthesis by increasing glucose uptake and

stimulation of translocation of glucose receptors to the cell surface. PAI-1 is known to cause vascular thrombosis, and its levels are strongly correlated with factors associated with metabolic syndrome such as dyslipidemia, visceral obesity, and insulin resistance. It is also known to be stimulated by TNF-α which is also associated with insulin resistance (Fruhbeck et al. 2001; Gil-Campos et al. 2004; Juge-Aubry et al. 2005).

Adipocytokines can have endocrine, autocrine, or paracrine function depending on their identity and target tissues. They enable adipose tissue to regulate metabolism and energy homeostasis (Mohamed-Ali et al. 1998). Boucher *et al*, 2005, found that adipose tissue exhibits strong heterogeneity in gene expression depending on the anatomical location. They concluded that depot-specific variations in adipocytokine expression may contribute to obesity-associated diseases (Boucher et al. 2005). Researchers have begun to recognize that many of the consequences of obesity, such as diabetes, hypertension, and cardiovascular disease are greatly influenced by the actions of these adipocytokines (Ouchi et al. 2001; Koerner et al. 2005). Therefore, the actions and targets of these adipocytokines are being studied as possible therapeutic targets for the prevention of obesity and its associated disorders.

Adipose Tissue and Muscle Tissue Interaction - The interactions that occur between skeletal muscle and adipose tissue play a significant role in growth and development which include utilization of energy substrates, muscle growth, and energy storage. As the main location for storage of energy for metabolism, adipose tissue releases energy at necessary times for use by muscle and other parts of the body. Free fatty acids serve as a highly energetic fuel, important signaling molecules, and their availability is an important factor influencing glucose utilization in muscles. The adipose

tissue has a role in buffering the fatty acid fluxes that occur during the post-prandial period or during fasting. If the buffering function is impaired, then other tissues, such as liver or muscle, are exposed to excessive fluxes of lipid fuels which may lead to impairment of normal regulation mechanisms (Cahova et al. 2006). The adipocytokines released from fat also play a large role in utilization and partitioning of energy substrates. The anatomical location of the fat depot can also determine the actions the adipose tissue has on the muscle. Fat depots which are located close to muscle, such as intermuscular or subcutaneous depots, have a different effect than those located further away, such as mesenteric or peri-renal depots. The utilization of lipid, carbohydrate, and protein for energy depends on many intrinsic and extrinsic factors such as temperature, stress, nutritional status, age, sex, and exercise. Insulin has been reported to inhibit lipolysis by enzymes such as lipoprotein lipase (LPL) and to induce the movement of fatty acid transport proteins (FATP/CD36) and receptors to the plasma membrane. The translocation of these transporters increases the flux of fatty acids into the cells and favors the storage of triacylglycerol in intermuscular fat depots and leading to a decrease in fatty acid oxidation. Leptin may counter these actions by increasing diacylglycerol synthesis, reducing insulin-stimulated insulin receptor substrate (IRS-1) associated phosphatidylinositol 3-kinase (PI3-kinase) activity, and inhibiting glucose transport and phosphorylation (Kokta et al. 2004).

Adipose tissue releases free fatty acids into circulation following lipolysis, and high levels of circulating free fatty acids have been associated with obesity and type 2 diabetes. Visceral fat releases these free fatty acids directly into the hepatic portal vein; consequently, high levels of fatty acids accumulate in the liver and can contribute to

dyslipidemia and hepatic insulin resistance leading to increased gluconeogenesis. In the skeletal muscle, the high concentrations of circulating free fatty acids can lead to fatty acids being preferentially oxidized over glucose leading to increased serum glucose levels (Arner 2002). Several different theories have been proposed for the mechanism by which high circulating levels of free fatty acids induce insulin resistance. Randle *et al*, 1963, proposed that increased free fatty acid levels increase mitochondrial acetyl-CoA/CoA ratios which inhibits pyruvate dehydrogenase activity and increases citrate levels. The increased citrate level in turn inhibits phosphofructokinase activity which leads to increased glucose-6-phosphate concentrations. The high concentration of glucose-6-phosphate inhibits hexokinase and reduces glucose transport and phosphorylation (Randle et al. 1963). Griffin *et al*, 1999, found that acute elevation of plasma FFA concentrations reduced the rate of muscle glycogen synthesis and the relative rate of muscle glucose oxidation. These findings indicated a defect in glucose transport and phosphorylation. PI3-kinase regulates the translocation of GLUT-4 in muscle, and elevation of circulating FFA levels was associated with decreased PI3-kinase activity. The decrease in PI3-kinase activity may have occurred as a result of reduced IRS-1 phosphorylation (Griffin et al. 1999). Roden *et al*, 1996, reported that elevation of free fatty acid concentrations caused inhibition of glucose phosphorylation and transport which lead to a reduction in the rate of glucose oxidation and muscle glycogen synthesis. The reduction in insulin-induced glucose transport/phosphorylation is similar to the insulin resistance seen in type 2 diabetes (Roden et al. 1996). Boden *et al*, 2001, found that intramyocellular triglyceride increased dose-dependently with increasing plasma FFA concentrations and the triglyceride accumulation led to an increase in whole-body

insulin resistance (Boden et al. 2001). Hulver *et al*, 2003, examined the oxidation rate of palmitate in skeletal muscle of extremely obese, overweight/obese, and normal individuals. They discovered that palmitate oxidation was lower, palmitate incorporation into intramyocellular triacylglycerols was higher, and long-chain fatty acyl-CoAs were significantly higher in the muscle tissue of extremely obese individuals. This group theorized that the reduced oxidation of fatty acids may result in the accumulation of intramyocellular triacylglycerols in skeletal muscle of extremely obese subjects, and this could then lead to development of insulin resistance (Hulver et al. 2003). Dobbins *et al*, 2001, found that an increase in dietary fat intake caused a strong reduction in whole-body insulin sensitivity which was reflected in impairment of both insulin-medicated glucose disposal and suppression of endogenous glucose production during the hyperinsulinemic-euglycemic clamp (Dobbins et al. 2001). Other researchers summarized that the most important mechanisms underlying the development of insulin resistance is the impaired ability of skeletal muscle to oxidize fatty acids due to elevated glucose oxidation and metabolic inflexibility. Metabolic inflexibility is defined as the impaired ability to switch between utilization of glucose and fat for energy in response to homeostatic signals. Researchers describe studies which show a decrease in fat oxidation with increases in glucose availability. Intramyocellular accumulation of fatty acid metabolites and long chain acyl-CoA (LC-AcCoA) induces insulin resistance. The accumulation of LC-AcCoAs decreases phosphorylation of insulin receptor substrates. As a result, PI3-kinase is not activated and this leads to decreased activation of glucose transport in both muscle and adipose tissue (Cahova et al. 2006).

ADIPONECTIN

Adiponectin was discovered between 1995-1996 by several different research groups and is consequently known by several different names; Acrp30, apM1, GBP28, and adipoQ. It is a 30-kDa adipocytokine hormone secreted primarily by the adipose tissue. Recently, it has been reported that the adiponectin gene is also expressed by the liver, pituitary gland, diencephalon, kidney, and skeletal muscle (Scherer et al. 1995; Hu et al. 1996; Maeda et al. 1996; Nakano et al. 1996; Maddineni et al. 2005). There is significant homology between the adiponectin gene of humans, mice, rats, cows, and monkeys (Berg et al. 2002). Adiponectin has been mapped to an area of chromosome 3q27 which contains a diabetes and metabolic syndrome susceptibility locus which is associated with an increase in total adiposity, abdominal obesity, and insulin resistance (Kissebah et al. 2000; Takahashi et al. 2000). Adiponectin is a relatively abundant serum protein and can account for between 0.01-0.03% of total serum protein in humans (Scherer et al. 1995; Berg et al. 2002; Stumvoll et al. 2002). Qi *et al*, 2004, demonstrated that adiponectin is able to cross the blood-brain barrier from the serum and enter the cerebrospinal fluid. Once it enters the spinal fluid, adiponectin decreases body weight by acting in the brain to increase energy expenditure (Qi et al. 2004). Adiponectin has been getting a lot of attention from researchers because increased serum levels of this protein are associated with increased insulin sensitivity, increased fatty acid oxidation, and decreased hepatic glucose production (Berg et al. 2001; Combs et al. 2001; Fruebis et al. 2001; Berg et al. 2002).

Structure - The protein is comprised of 244 amino acids and has an amino-terminal signal sequence, a collagenous region, and a globular domain at the carboxylic

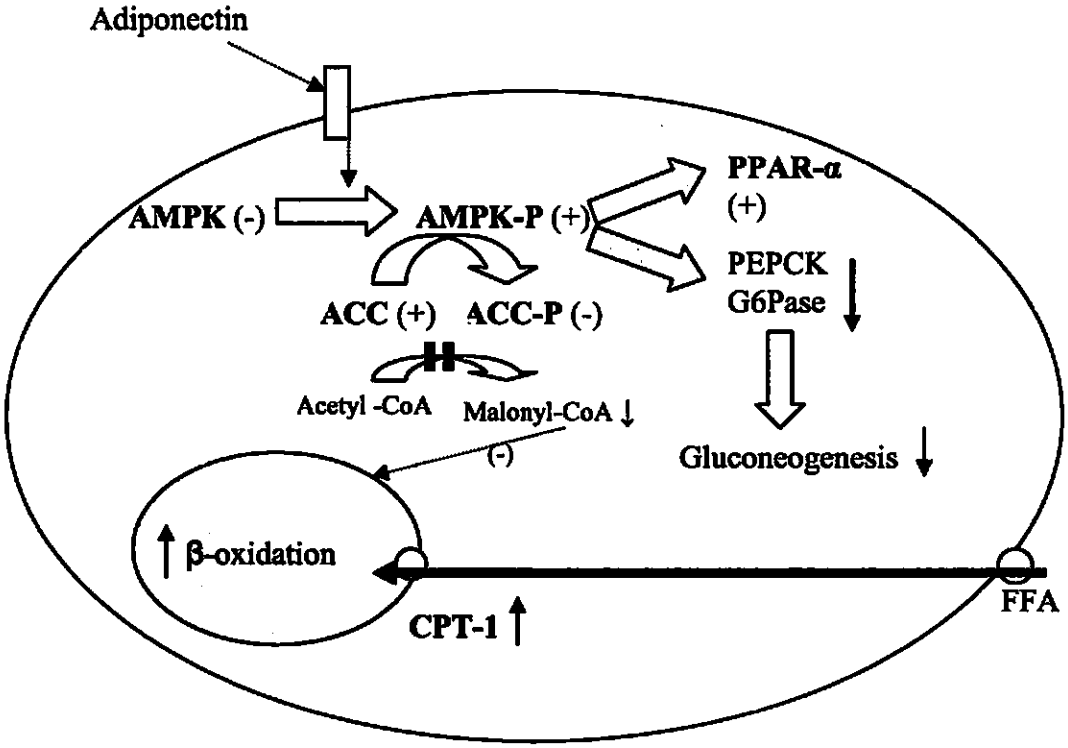
acid-terminus (Scherer et al. 1995; Hu et al. 1996; Maeda et al. 1996). The globular domain of adiponectin shares homology with similar domains of collagens VIII and X, and complement factor C1q. Members of this family share the characteristic of forming homotrimers or heterotrimers between different isoforms, such is the case with C1q. Full-length adiponectin (fAd) is secreted primarily by adipocytes and undergoes post-translational modification to form globular adiponectin (gAd). Once secreted, the C-terminal region is cleaved off, and the globular domain is able to interact with other globular domains to form a tightly associated homotrimer. The homotrimer can then interact with other trimers to form hexamers and higher molecular weight complexes of 12-18 subunits by linking via disulfide bonds at cysteine-39. Both the hexamers and higher molecular weight complexes are stable and do not spontaneously interconvert while present in serum (Shapiro et al. 1998; Pajvani et al. 2003).

In order to investigate the possibility of different actions of globular (gAd) and full-length adiponectin (fAd) in the body, one study injected both forms and observed different target sites for gAd and fAd (Yamauchi et al. 2002). The study found that gAd had a higher binding affinity to the skeletal muscle membranes and had a more pronounced effect on AMP-activated protein kinase (AMPK) activation in the skeletal muscle than fAd, but both forms of adiponectin had some degree of effect *in vitro* and *in vivo*. The researchers also found that fAd had a higher binding affinity for the hepatocyte membranes, and only fAd was able to activate and induce phosphorylation of AMPK *in vitro* and *in vivo*. The different responses in skeletal muscle and liver to the fAd and gAd indicated that there may be two different receptors functioning at the two sites (Yamauchi et al. 2002).

Adiponectin Gene Regulation - The molecular mechanisms that regulate the expression of the adiponectin gene are still under investigation and poorly understood. A PPAR-γ response element has been found in the promoter region of adiponectin and TZDs, which are PPAR-γ agonists, have been shown to increase adiponectin expression (Maeda et al. 2001). TNF-α has been shown to inhibit expression of adiponectin (Fasshauer et al. 2002). Kita *et al*, 2005, reported that the inhibitory effect TNF-α has on adiponectin transcription may be due to the inactivation of CCAAT/Enhancer binding proteins (C/EBP) (Kita et al. 2005). C/EBPs and PPAR-γ are key regulators of adipocyte differentiation and have been reported to activate the promoter region of adiponectin and in some cases be required for full activation of adiponectin gene transcription (Park et al. 2004; Qiao et al. 2005). Neschen *et al*, 2006, reported that dietary fish oil, a possible PPAR-γ ligand, increases plasma adiponectin concentrations and that transcription of the adiponectin gene was increased in the epididymal fat mice fed fish oil (Neschen et al. 2006). These are a few of the proposed mechanisms for the activation and inhibition of adiponectin gene expression which exist, but many factors are still under investigation.

Adiponectin Alters Carbohydrate Metabolism - Adiponectin has been known to ameliorate insulin resistance by enhancing hepatic insulin action and by decreasing hepatic glucose production. See Figure 1 for mechanisms of action of adiponectin in hepatocytes (Gil-Campos et al. 2004; Dyck et al. 2006). Injection of adiponectin into wild-type and diabetic mouse models (both type 1 and type 2 diabetes) led to decreased serum glucose levels. No significant change in insulin levels was noted, but glucagon levels were increased. Since injection of adiponectin produced a decrease in basal

FIGURE 1. Actions of adiponectin in hepatocytes



Adapted from Dyck *et al*, 2006. AMPK: AMP-kinase; ACC: Acetyl-CoA carboxylase; PEPCK: Phosphoenolpyruvate carboxykinase; G6Pase: Glucose-6-phosphatase; CPT-1: Carnitine palmitoyl transferase-1; FFA: Free fatty acids. This figure portrays the signal transduction mechanism by which adiponectin increases fatty acid oxidation and decreases hepatic gluconeogenesis.

glucose levels without increasing insulin levels, researchers concluded that adiponectin itself was able to suppress hepatic glucose production (Berg et al. 2001). Combs *et al*, 2001, demonstrated that short-term intravenous infusion of adiponectin into conscious mice produced a significant decrease in hepatic glucose production. They also noted significantly lower activity of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) in mice which were infused with adiponectin. The decrease in activity of PEPCK was accompanied by significantly lower mRNA expression of PEPCK and glucose-6-phosphatase (G6Pase) in liver tissue (Combs et al. 2001). Yamauchi *et al*, 2002, independently confirmed that administration of fAd, which is primarily active in the liver, reduced the expression levels of the PEPCK and G6Pase in mice. Activation of AMPK was necessary for adiponectin to reduce the activity of the gluconeogenic enzymes. This study also demonstrated that adiponectin was able to increase glucose uptake through a PI3-kinase independent pathway (Yamauchi et al. 2002). The same group of researchers created transgenic mice with elevated plasma concentrations of gAd which showed reduced triglyceride content in skeletal muscle and in the liver even when on a high-fat diet. These mice showed improved insulin sensitivity and increased glucose tolerance even though there was no significant change in body weight. The transgenic mice with elevated gAd were mated with $Lep^{ob/ob}$ mice and the offspring showed a significant increase in food intake over non-transgenic/$Lep^{ob/ob}$ littermates, but there were no differences in body weight between the two groups. This indicates that the transgenic/$Lep^{ob/ob}$ mice had an increased energy expenditure which prevented excess weight gain even though they consumed more calories. The transgenic/$Lep^{ob/ob}$ mice showed significantly increased insulin sensitivity and glucose tolerance along with

increased insulin immunoreactivity and insulin content. There was amelioration of diabetes in the transgenic ob/ob mice fed a high-fat or high-carbohydrate diet. This was most likely due to the increased expression of molecules involved in fatty acid oxidation such as acetyl-CoA oxidase and increased fatty acid oxidation in the skeletal muscle. The transgenic/Lep$^{ob/ob}$ mice showed decreased serum and tissue triglyceride content as well as decreased levels of serum free fatty acids (Yamauchi et al. 2003).

Adiponectin also affects glucose uptake into adipocytes. Treatment of mature adipocytes with gAd increased basal glucose uptake and enhanced insulin-stimulated glucose uptake in a dose-dependent manner (Morris et al. 2006). gAd was also able to decrease the inhibitory effect TNF-α had on insulin-stimulated glucose uptake. TNF-α is able to initiate the inflammatory cascade in obesity and insulin resistance states. TNF-α and adiponectin produce opposite effects in the body and are thought to serve as inhibitors of each other (Morris et al. 2006). TNF-α has also been shown to mediate insulin resistance by decreasing activity of IRS-1 and GLUT-4, inhibit LPL, and display increased levels in obesity (Mohamed-Ali et al. 1998; Hotta et al. 2001). gAd had no direct effect on insulin-receptor and IRS-1 phosphorylation which indicates the increase in glucose uptake is through an insulin-independent mechanism. Furthermore, it was noted that when adipocytes were incubated with gAd, phosphorylation of AMPK and ACC were increased indicating that AMPK may mediate the increase in glucose uptake into adipocytes (Wu et al. 2003).

Adiponectin Increases Fatty Acid Oxidation - Along with its ability to decrease the activity of hepatic gluconeogenic enzymes, adiponectin is thought to improve insulin sensitivity by decreasing the concentration of circulating triglycerides and free fatty

acids. One method adiponectin is thought to improve blood lipid levels is by increasing the levels of fatty acid oxidation. Freubis *et al*, 2001, reported that injection of gAd into mice after feeding a high-fat/high-sucrose meal significantly decreased the levels of plasma free fatty acids, glucose, and triglycerides compared to mice injected with a placebo. Injection of fAd only slightly improved plasma free fatty acids and glucose but had no significant effect on plasma triglyceride levels. The investigators deduced that gAd was promoting cellular uptake of free fatty acids and that gAd led to a significant increase in oleate oxidation in isolated EDL, soleus muscles, and C2C12 muscle cell culture (Fruebis et al. 2001). Another study found that injection of adiponectin into mice prevented the increase in serum triglycerides which typically occurs after feeding (Berg et al. 2001). Adiponectin is also able to accelerate free fatty acid clearance by increasing fatty acid transport protein 1 (FATP-1) mRNA expression (Maeda et al. 2002). Adiponectin also increases expression of FATP-1 by counteracting TNF-α's inhibitory effect on FATP-1 (Maeda et al. 2002). Yamauchi *et al*, 2002, found that both gAd and fAd increased phosphorylation of AMPK and ACC in C2C12 myocytes and skeletal muscle *in vivo* in a dose-dependent manner, but gAd produced a more significant effect. In order to better understand the mechanism by which adiponectin activates AMPK, the research group measured the cellular AMP content, an activator of AMPK, in C2C12 myocytes and soleus muscle cells. They found that the concentration of AMP was increased by 2-fold after treatment with adiponectin. The researchers concluded that the activation of AMPK seen after treatment with adiponectin may result because of an increase in the cellular content of AMP (Yamauchi et al. 2002). In a study using cell culture of skeletal muscle from lean and obese subjects, it was found that gAd stimulated

AMPK and ACC phosphorylation and subsequently stimulated fatty acid oxidation in lean skeletal muscle, but the effects seen in obese skeletal muscle were much lower. The muscle cell cultures from obese subjects required much higher levels of gAd to induce fatty acid oxidation. This is discouraging because adiponectin levels are already typically lower in obese individuals, and an increase in the amount of adiponectin needed to stimulate obese muscle would further depress fatty acid oxidation (Chen et al. 2005). Tomas *et al*, 2002, showed that incubation of EDL muscle with gAd led to a 2-fold increase in the activity of AMPK and that glucose transport in the absence of added insulin was increased by 50%. After the serum AMPK levels were increased, ACC phosphorylation and fatty acid oxidation also increased. *In vivo* studies done on C57BL/6J mice showed similar results; the AMPK and ACC phosphorylation increased, and a decrease in serum malonyl-CoA levels followed. The sequential events of AMPK phosphorylation and activation, ACC deactivation, decline in malonyl-CoA levels, and an increase in fatty acid oxidation indicate that adiponectin produces its effects on serum lipid levels by first acting on AMPK (Tomas et al. 2002).

AMP-activated protein kinase - Several studies have shown that adiponectin stimulates beta-oxidation and glucose uptake via AMPK activation (Hayashi et al. 2000; Yamauchi et al. 2001; Park et al. 2002; Tomas et al. 2002; Yamauchi et al. 2002; Kokta et al. 2004). AMPK is ubiquitous and senses the amount of fuel, in the form of ATP, present in tissues or cells. Cells monitor the AMP/ATP ratio. In instances where there is a decline in energy levels, an increase in the AMP/ATP ratio, AMPK is phosphorylated by an AMPK kinase (Tomas et al. 2002). Once AMPK is activated, it in turn phosphorylates a variety of proteins to increase ATP generation, such as acetyl CoA

carboxylase (ACC) and malonyl-CoA decarboxylase (MCD). ACC is the rate limiting enzyme in the formation of malonyl-CoA and phosphorylation de-activates it. MCD is activated by phosphorylation and it degrades malonyl-CoA. The combination of deactivation of ACC and activation of MCD decreases the cellular concentration of malonyl-CoA. Malonyl-CoA is an inhibitor of carnitine palmitoyl transferase 1 (CPT-1). CPT-1 is the enzyme which regulates the rate of transfer of long chain fatty acyl-CoA into the mitochondria for oxidation. High concentrations of malonyl-CoA in the body are linked to insulin resistance because it restricts the entrance of long chain fatty acyl-CoAs into the mitochondria. The fatty acyl-CoAs would accumulate in the cytosol and muscle as triglycerides, and this fatty acid accumulation could contribute to insulin resistance (Ruderman et al. 1999; Park et al. 2002). Once ACC is phosphorylated and inactivated by AMPK, concentrations of malonyl-CoA decrease, and since CPT-1 is no longer inhibited, fatty acid oxidation is stimulated. AMPK is able to produce effects in many tissues such as liver, skeletal muscle, adipocytes, and pancreatic islets (Winder et al. 1999). Adiponectin has been shown to increase AMPK activity and subsequently increasing fatty acid oxidation in the mitochondria both *in vitro* and *in vivo* (Yamauchi et al. 2001; Tomas et al. 2002; Yamauchi et al. 2002).

ADIPONECTIN AND CHRONIC DISEASE

There has been a strong negative correlation shown between plasma adiponectin levels and BMIs of men and women. Obesity tends to increase the plasma concentrations of most proteins produced by adipose tissue because of the increase in total fat mass, but plasma levels of adiponectin were found to be much lower in obesity (Arita et al. 1999). Plasma adiponectin concentration was reported to be significantly lower in individuals

with essential hypertension, coronary artery disease (CAD), and type 2 diabetes, especially in people with type 2 diabetes and CAD, than in the control subjects. (Ouchi et al. 1999; Hotta et al. 2000; Adamczak et al. 2003).

Obesity & Type 2 Diabetes - Many murine models of obesity and type 2 diabetes have shown low levels of adiponectin which coincide with development of insulin resistance. Overfeeding and obesity lead to decreased serum adiponectin concentrations, but calorie restriction can increase adiponectin levels and increase insulin sensitivity (Maddineni et al. 2005). Significantly lower levels of adiponectin were observed in fat tissues from obese mice and obese humans (Hu et al. 1996). Weyer *et al*, 2001, confirmed previous findings that obesity and type 2 diabetes are closely associated with low plasma adiponectin levels. They found that plasma adiponectin negatively correlated with BMI, percent body fat, waist-to-thigh ratio, fasting plasma insulin, and glucose tolerance. They determined that plasma adiponectin concentration was most closely related to insulin sensitivity and fasting insulinemia rather than adiposity and glycemia (Weyer et al. 2001). Degawa-Yamauchi *et al*, 2005, found that expression of adiponectin mRNA negatively correlated with the BMI of individuals. It was also reported that a reduction in serum adiponectin also highly correlated with increasing accumulation of intra-abdominal fat mass (Degawa-Yamauchi et al. 2005).

In situations where over-nutrition exists, such as a high-fat diet, reduced mRNA levels of adiponectin in adipose tissue and corresponding lower serum adiponectin levels have been observed in concert with hyperglycemia and hyperinsulinemia. When globular adiponectin was administered intravenously, hyperglycemia and hyperinsulinemia were ameliorated. At the same time, increased expression of molecules involved in fatty acid

transport and combustion such as acetyl-CoA oxidase, uncoupling protein-2, and fatty acid translocase/CD36 was observed. This led to a decrease in muscle triglyceride content. The decrease in tissue triglyceride levels may improve insulin signal-transduction and thus reverse the insulin resistance of obese mice. Serum adiponectin levels of genetically obese KKAy mice on a high-fat diet were much lower than the adiponectin levels of KKAy mice on a high-carbohydrate diet, and administration of adiponectin was able to ameliorate the insulin resistance and high triglyceride levels in these mice (Yamauchi et al. 2001). Decreased plasma adiponectin has been shown to correspond with or precede insulin resistance in both murine and primate models (Hotta et al. 2001; Yamauchi et al. 2001). Rhesus monkeys are good primate models for human type 2 diabetes since they spontaneously develop obesity and subsequent diabetes. Hotta *et al*, 2001, were able to detect metabolic changes which preceded the development of diabetes in these monkeys. The plasma concentration of adiponectin in obese monkeys was significantly lower than levels in lean monkeys and stayed significantly lower after the monkeys developed diabetes. The reduction of plasma adiponectin coincided with the early stages of obesity, the decrease in insulin sensitivity, and development of insulin resistance (Hotta et al. 2001).

One class of drugs used to treat diabetes is the thiazolidinediones (TZDs). These drugs are PPAR-γ agonists which have been reported to increase the number of small adipocytes and induce normalization of plasma lipids, leading to the amelioration of insulin resistance (Okuno et al. 1998). Maeda *et al*, 2001, found that TZDs are able to enhance the expression and secretion of adiponectin by activation of its promoter. They also found that TNF-α had a suppressive effect on adiponectin expression, but that TZDs

were able to alleviate this suppression (Maeda et al. 2001). Inukai *et al*, 2005, found that adiponectin mRNA expression in epidydimal fat from obese mice was significantly decreased compared to lean mice, but treatment with pioglitazone, a TZD, improved adiponectin mRNA expression and protein secretion (Inukai et al. 2005). Phillips *et al*, 2003, also found that plasma adiponectin levels were highest in lean, non-diabetic subjects and significantly lower in obese type 2 diabetics. After 3-4 months of treatment with troglitazone, a TZD, there was a significant increase in the average serum adiponectin level of these subjects and the change in serum adiponectin levels was positively correlated with improvement in whole-body insulin action. Treatment with troglitazone also improved the circulating lipid profile with increases in HDL (Phillips et al. 2003). Iwaki *et al*, 2003, reported the existence of a PPAR-response element (PPRE) in the human adiponectin promoter. The PPAR-γ/RXR heterodimer was found to bind specifically to the human adiponectin PPRE in the promoter region. This discovery partially explains the increase in serum adiponectin levels seen when patients are treated with PPAR-γ agonists like TZDs (Iwaki et al. 2003).

Cardiovascular Disease - Similar to the decreased adiponectin levels observed in obesity and type 2 diabetes, serum adiponectin levels have been shown to be lower in patients with cardiovascular disease. Plasma adiponectin levels are especially low in people with type 2 diabetes and coronary artery disease. Serum triglyceride level was negatively correlated, and serum HDL was positively correlated with the plasma adiponectin levels in participants of one study (Hotta et al. 2000). Ouchi *et al*, 1999, also found that plasma adiponectin levels were much lower in patients with coronary artery disease compared to age- and BMI-adjusted controls. TNF-α has been reported to

enhance adhesion of the human monocytic cell line THP-1 cells to human aortic endothelial cells (HAEC), and this action was dose-dependently suppressed by incubation of the HAECs with adiponectin. Dose-dependent suppression of TNF-α-induced surface expression of vascular cell adhesion molecule-I (VCAM-I), endothelial-leukocyte adhesion molecule-I (E-selectin-I), and intracellular adhesion molecule-1 (ICAM-1) was also observed when HAECs were pre-treated with adiponectin. These molecules have been detected in human atherosclerotic lesions, and abnormal leukocyte adhesion to the vascular wall is considered to be crucial for the development of atherosclerosis. The main finding of this study was that physiological concentrations of adiponectin were able to significantly inhibit TNF-α-induced monocyte adhesion and adhesion molecule expression (Ouchi et al. 1999). TNF-α stimulates VCAM-I, E-selectin-I, and ICAM-I through activation of nuclear transcriptional factor- κB (NF-κB). Ouchi *et al*, 2000, also reported that adiponectin was found to suppress TNF-α-induced NF-κB activation through a cAMP-dependent pathway. This caused researchers to believe that adiponectin acts as an endogenous modulator of the endothelial inflammatory response (Ouchi et al. 2000). The decrease in plasma adiponectin seen in patients with coronary artery disease may directly correlate with the development of vascular disease. The same research group conducted further studies related to the ability of adiponectin to inhibit TNF-α-induced events which lead to coronary artery disease. Treatment of macrophages with physiological concentrations of adiponectin significantly reduced cholesteryl ester contents; this indicates that adiponectin is able to suppress the transformation of macrophages to foam cells (Ouchi et al. 2000). Treatment with adiponectin was also able to dose-dependently suppress type I and II class A macrophage scavenger receptor

(MSR) mRNA expression levels. Adiponectin was also found to be abundant in the endothelium and sub-endothelial space in lesions of injured human aorta. This indicated that adiponectin may accumulate in the vascular wall when the endothelium is damaged and modulate the transformation of macrophages to foam cells transformation (Ouchi et al. 2001). Apo-E deficient mice exhibit hypercholesterolemia and spontaneously develop severe atherosclerosis. Yamauchi *et al*, 2003, mated Apo-E deficient mice with transgenic mice expressing high levels of gAd and found that the offspring who were positive for the transgene developed lesions which were significantly smaller than those in non-transgenic littermates. The over-expression of adiponectin also ameliorated hyperlipidemia without changing body weight, lipoprotein profiles, levels of serum total cholesterol, HDL cholesterol, free fatty acids, or triglycerides. This indicated to researchers that gAd may have a protective effect which directly acts on the vascular wall and/or macrophages rather than indirectly improving circulating lipids and proteins (Yamauchi et al. 2003). Adiponectin has demonstrated ability to inhibit foam cell formation, down-regulate vascular adhesion molecules and immune cell activation, and reduce cholesteryl ester contents and lipid droplets in human monocyte-derived macrophages (Hotta et al. 2000; Ouchi et al. 2001; Adamczak et al. 2003; Koerner et al. 2005; Okamoto et al. 2006). Hypoadiponectinemia has been associated with low HDL cholesterol, high TNF-α concentrations, high plasma triglycerides, obesity, and type 2 diabetes (Weyer et al. 2001; von Eynatten et al. 2004). Low levels of plasma adiponectin are also associated with patients who have essential hypertension irrespective of gender. A significant negative correlation was found between blood pressure and plasma adiponectin concentration (Adamczak et al. 2003).

ADIPONECTIN RECEPTORS

Yamauchi *et al*, 2002, are credited with discovering a differential response to gAd and fAd. They were also able to isolate cDNA for the two different types of adiponectin receptors. The receptors are AdipoR1 (42.4 kDa) and AdipoR2 (35.4 kDa), and found that human and mouse adiponectin receptors shared up to 96% homology. It was also noted that AdipoR1 was ubiquitously expressed, but most predominantly in the skeletal muscle and AdipoR2 was primarily expressed in the liver. The two receptors are structurally similar and contain seven trans-membrane domains. Expression of AdipoR1/R2 in C2C12 myoctyes was associated with increased PPAR-α ligand activity, fatty acid oxidation, and glucose uptake. Suppression of AdipoR1 expression with siRNA greatly reduced the binding of gAd but only slightly reduced the binding of fAd. It also greatly decreased PPAR-α ligand activity, fatty acid oxidation, and glucose uptake. Conversely, suppression of AdipoR2 expression with siRNA significantly reduced the binding of fAd but only moderately reduced the binding of gAd and only partially reduced PPAR-α ligand activity and fatty acid oxidation. A Scatchard plot analysis showed that AdipoR1 is a high-affinity receptor for gAd but a very low-affinity receptor for fAd, and that AdipoR2 is an intermediate affinity receptor for both gAd and fAd. Both receptors mediate the actions of adiponectin to increase fatty acid oxidation and glucose uptake (Yamauchi et al. 2003).

Adiponectin Receptor Expression - Due to the relatively recent discovery of adiponectin receptors, the factors regulating their expression have not been completely elucidated. The mechanisms for signaling of adiponectin receptors are still under investigation, as are the factors which control their expression. Inukai *et al*, 2005, found

that insulin has an inhibitory effect on AdipoR1 both *in vivo* and *in vitro*, and its expression was also significantly suppressed in genetically obese and diabetic mice, while AdipoR2 expression remained unchanged (Inukai et al. 2005). Fang *et al*, 2005, found that both hyperglycemia and hyperinsulinemia caused a significant reduction in AdipoR1 expression, but that hyperinsulinemia increased AdipoR2 expression in muscle tissue of rats. They also found that gAd, but not fAd, increased glucose uptake mainly by increasing the amount of GLUT4 located on the cell membrane surfaces. Although both gAd and fAd had the ability to increase fatty acid oxidation, hyperglycemia significantly decreased fatty acid oxidation levels, and hyperinsulinemia reduced basal fatty acid oxidation levels. This group noted that myoblasts express six times more AdipoR1 mRNA than AdipoR2 mRNA (Fang et al. 2005). Another feature of AdipoR1 is that it has been shown to interact with insulin receptors which enhance insulin signal transduction and possibly decrease insulin resistance (Koerner et al. 2005). Several other research groups found AdipoR1 mRNA expression to be significantly greater than AdipoR2 mRNA expression in muscle cells (Chen et al. 2005; Fang et al. 2005).

Chen *et al*, 2005, found that adiponectin receptor expression is not significantly different in muscle cells from lean and obese subjects, but Kadowaki *et al*, 2005, found that expression of both adiponectin receptors were greatly decreased in muscle and adipose tissue of insulin resistant $Lep^{ob/ob}$ mice (Chen et al. 2005; Kadowaki et al. 2005). Tsuchida *et al*, 2004, found significantly higher levels of AdipoR1 and AdipoR2 mRNA expression in liver and skeletal muscles after fasting, and the levels were restored to normal after re-feeding. Plasma insulin levels can also be affected by fasting and re-feeding. Insulin levels are typically decreased with fasting and tend to increase with

feeding. Because of the opposite trends of adiponectin receptor expression and insulin levels, researchers hypothesized that insulin has the ability to inhibit the expression of AdipoR1 and AdipoR2. They elucidated that high levels of insulin significantly decreased the mRNA expression of both adiponectin receptors. They also found that expression of both adiponectin receptors was significantly down-regulated in $Lep^{ob/ob}$ mice compared to control mice. Binding affinity of adiponectin was much lower in skeletal muscle of $Lep^{ob/ob}$ mice than in wild-type mice. It was also noted that although adiponectin was able to activate AMPK in skeletal muscle from wild-type mice, it was unable to activate AMPK in the skeletal muscle of $Lep^{ob/ob}$ mice (Tsuchida et al. 2004).

Similarly, if chronic overfeeding leads to increased insulin levels, based on the results of this study, it makes sense that there would be decreased expression of adiponectin receptors in skeletal muscle and hepatocytes. The decrease in receptors would lead to less adiponectin binding sites and consequently less activation of the AMPK pathway, decreased fatty acid oxidation, and decreased glucose utilization would result. In contrast to the effect insulin had on mRNA expression of AdipoR1/R2 in the previous study, another group found no significant effect of insulin on AdipoR1 mRNA expression (Staiger et al. 2004). They did find that myotubes expressed AdipoR1 1.8-fold higher than AdipoR2, and the expression levels of AdipoR1/R2 are positively correlated with each other and also correlated to plasma triglyceride concentrations (Staiger et al. 2004). Tsuchida *et al*, 2005, found that administration of PPAR-α and PPAR-γ agonists enhanced the action of adiponectin in white adipose tissue by increasing adiponectin levels and increasing the expression of its receptors (Tsuchida et al. 2005). Another group found that AdipoR1 and AdipoR2 had different mechanisms for

improving diabetes in leptin deficient ($Lep^{-/-}$) mice. They found that AdipoR1 significantly increased activation of AMPK in the liver, but AdipoR2 did not. The activation of AMPK in the liver also was accompanied by decreased expression of gluconeogenic enzymes. AdipoR2 was found to significantly increase the expression of the gene encoding glucokinase in the liver which is involved in glucose uptake, but AdipoR1 did not. AdipoR2 activation increased the expression of the gene encoding PPAR-α and its target genes Acox1 and Ucp2, but activation of AdipoR1 had little effect on these genes. These results indicate that AdipoR2 may be more involved in the activation of PPAR-α than AdipoR1. Mice which had either AdipoR1 or AdipoR2 genes "knocked-out" showed increased plasma glucose and insulin levels indicating these mice developed insulin resistance. The combination of AdipoR1/R2 knock-out mice significantly reduced adiponectin binding and effects. Consequently, these mice appeared to be significantly more insulin resistant and glucose intolerant than mice which had only the AdipoR1 or AdipoR2 gene knocked-out (Yamauchi et al. 2007). There are many factors which may affect the expression of adiponectin receptors AdipoR1 and AdipoR2, and these mechanisms may become future drug targets in order to improve chronic diseases such as diabetes and cardiovascular disease.

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS (PPAR-α, PPAR-γ)

PPARs are ligand-activated transcription factors which are part of the nuclear hormone receptor superfamily. They bind to DNA elements called PPAR response elements (PPREs) and form a heterodimer with 9-*cis*-retinoic acid receptors (RXRs) in the promoter region of many genes. The binding of these elements to the DNA sequence can either promote activation or induce repression of gene expression (Ferre 2004). Both

PPAR-α and PPAR-γ are able to lower circulating lipid concentrations and are therefore able to ameliorate insulin resistance via different mechanisms (Muoio et al. 2002).

PPAR-α - PPAR-α is widely expressed in the liver, muscle, kidney, and intestine, but is also expressed to a small degree in adipose tissue. The activation of PPAR-α stimulates uptake, binding, and oxidation of fatty acids in mitochondria, peroxisomes, and microsomes resulting in decreased tissue content of free fatty acids (Ye et al. 2001; Kersten 2002). Natural ligands for PPAR-α include unsaturated fatty acids and saturated fatty acids, but saturated fatty acids have a lower affinity for PPAR-α (Ferre 2004). Synthetic ligands for PPAR-α include fibrates such as fenofibrate and clofibrate (Ferre 2004). In the liver, activation of PPAR-α stimulates the expression of fatty acid transport proteins and key enzymes involved in peroxisomal beta-oxidation. One possible target of PPAR-α is carnitine palmitoyl transferase I (CPT-I) which is the rate-limiting step of mitochondrial beta-oxidation (Ferre 2004). By stimulating pyruvate dehydrogenase kinase 4 (PDK4) gene expression, PPAR-α facilitates the phosphorylation of pyruvate dehydrogenase. The deactivation of pyruvate dehydrogenase by phosphorylation induces glucose carbon sparing in the skeletal muscle and favors the use of fatty acids as fuel (Ferre 2004). Ye *et al*, 2001, found that treatment of high-fat fed rats with PPAR-α agonists ameliorated whole-body insulin resistance. They found that the improved muscle insulin action was associated with a reduction in circulating and intramuscular long-chain acyl-CoAs. The decrease in muscle lipid accumulation may be the mechanism by which PPAR-α agonists improve insulin sensitivity (Ye et al. 2001). Muoio *et al*, 2002, also found that treating human skeletal muscle cells with a PPAR-α agonist increased fatty acid oxidation by 3-fold and also decreased fatty acid

esterification into myocyte triglyceride. They also noted a 45-fold increase in PDK4 mRNA levels which indicates that the gene is extremely responsive to PPAR-α activation. This strengthens Ye *et al*'s argument that an increase in PDK4 promotes fatty acid oxidation and leads to glucose carbon sparing (Muoio et al. 2002). Activation of PPAR-α in genetic models of insulin resistance has been found to increase insulin sensitivity and reduce accumulation of visceral fat (Guerre-Millo et al. 2000; Chou et al. 2002). This was primarily due to the role PPAR-α plays in the increase in oxidation of fatty acids in the liver and the heart which decreases levels of serum and tissue fatty acids (Ferre 2004). Studies have also shown that conjugated linoleic acid decreases body fat in animals (Whigham et al. 2000) and it has also been demonstrated that conjugated linoleic acid is a ligand for PPAR-α (Moya-Camarena et al. 1999). Consequently, researchers concluded that one mechanism by which conjugated linoleic acid has been able to decrease body fat is through activation of PPAR-α.

PPAR-γ - PPAR-γ is important regulator of gene expression and fat cell differentiation, triggering conversion of pre-adipocytes to adipocytes and regulating activation of lipopolysaccharide lipase, acyl-CoA synthase, fatty acid translocase (CD36), and fatty acid transport protein. PPAR-γ increases the number of small adipocytes which have been shown to be more insulin sensitive than large adipocytes (Rosen et al. 2001; Kersten 2002; Walczak et al. 2002; Ferre 2004). It stimulates storage of triglycerides by increasing the storage capacity of adipocytes and also increasing the fatty acid flow into the adipocytes. This reduces the amount of fatty acids present in the muscle and circulation which improves insulin sensitivity (Ferre 2004). Natural ligands for PPAR-γ include unsaturated fatty acids such as oleate, linoleate, eicosapentenoic, and arachidonic

acids, but they have been shown to have low affinity for PPAR-γ and scientists are still trying to identify natural, high-affinity PPAR-γ ligands (Rosen et al. 2000; Rosen et al. 2001; Ferre 2004). TZDs are synthetic, high-affinity PPAR-γ ligands used to treat diabetes which stimulate adipocyte differentiation, lower serum glucose levels, and improve the lipid profiles of people with type 2 diabetes. Fatty acid transporters FATP and CD36 are targets of PPAR-γ and stimulate the uptake of FFAs into adipose tissue thereby decreasing circulating concentrations of FFAs (Walczak et al. 2002). In a study by Vidal-Puig et al, 1996, it was shown that PPAR-γ mRNA expression levels were increased by feeding normal mice a high-fat diet, but expression levels in obese mice were not increased by the high-fat feeding (Vidal-Puig et al. 1996). This led to the conclusion that fatty acid ligands may be able to stimulate PPAR-γ only up to a certain threshold. Fu *et al*, 2003, proposed the idea that TGF-β may decrease PPAR-γ by decreasing its transcriptional activity (Fu et al. 2003). Zheng and Chen, 2007, demonstrated that TGF-β may have an inhibitory effect on PPAR-γ expression and activation. They found that TGF-β dose dependently reduced the activity of the PPAR-γ promoter (Zheng et al. 2007). They concluded that interruption of TGF-β signaling may alleviate the inhibitory effect on the PPAR-γ promoter region leading to an increase in PPAR-γ expression. If myostatin propeptide is able to bind and inactivate myostatin, a similar effect of increased PPAR-γ gene expression may occur.

Researchers have found that there is a PPAR-response element in the adiponectin promoter region so it correlates that the expression of adiponectin would be related to the expression of PPAR-γ (Iwaki et al. 2003). Other groups have also reported that stimulation of PPAR-γ by TZDs increases adiponectin expression and corresponds to

increased insulin-sensitivity in these subjects (Maeda et al. 2001; Phillips et al. 2003; Inukai et al. 2005). Muoio *et al*, 2002, also found that PPAR-α also increased expression of muscle CPT1 and MCD. CPT1 catalyses the rate-limiting step in the transport of fatty acids into the mitochondria, and MCD decreases concentrations of the CPT-1 inhibitor malonyl-CoA (Muoio et al. 2002). Both MCD and CPT-1 are involved in the mechanism by which adiponectin increases skeletal muscle fatty acid oxidation and therefore, it is likely that adiponectin may increase the activity of PPAR-α. Yamauchi *et al*, 2007, demonstrated that the over-expression of AdipoR2 increased the expression of the gene encoding PPAR-α and its target genes. Conversely, AdipoR2 KO mice showed a decrease in activity of PPAR-α and its target genes (Yamauchi et al. 2007).

MYOSTATIN

Myostatin, also known as growth/differentiation factor-8 (GDF-8), is a member of the transforming growth factor-β (TGF-β) superfamily. The TGF-β superfamily is made up of growth and differentiation factors which maintain tissue homeostasis. It is highly conserved among species and undergoes post-translational modification to yield the active form of the molecule. The sequences of murine, rat, human, porcine, chicken, and turkey myostatin are 100% identical at the C-terminal region which is active after undergoing proteolytic cleavage (McPherron et al. 1997). The active C-terminus region is 15kDa and has a highly conserved pattern of cysteine knots (Kocamis et al. 2002). The fact that there is considerable homology in the sequence of myostatin among many different animals indicates that both function and genetic sequence have been conserved. Myostatin produces its effects by specifically binding to ActRIIB. Smad proteins are the signal transducers which transmit signals from cell surface receptors to the nucleus where

they regulate transcription and translation (Lee et al. 2001; Tsuchida 2004). Myostatin is also negatively regulated by follistatin, which binds the C-terminal dimmer and prevents it from binding to receptors. Once myostatin is released from follistatin it is able to bind the ActRIIB receptors (Lee et al. 2001).

Effects of Myostatin - Animals which possess one or more mutations in the myostatin gene are much heavier than wild-type animals and display a significant increase in skeletal muscle mass. McPherron *et al*, 1997, found that the increase in skeletal muscle mass primarily resulted from muscle cell hyperplasia, but hypertrophy also contributed to the increase in total skeletal muscle mass (McPherron et al. 1997). Therefore, adult mice which have a mutation in the myostatin gene are up to 25-30% heavier than wild-type littermates, and their individual muscles can weigh 2-3 times as much, indicating that myostatin is a negative regulator of skeletal muscle growth (Lee et al. 1999). Significant differences in food intake were not observed between myostatin knockout mice and wild-type littermates at most stages of development. Body weights were significantly higher at most ages in myostatin knockout mice although fat-pad mass was lower in knockout mice than the wild-type mice. The increase in weight seen in the myostatin knockout mice was attributed to their greater skeletal muscle mass (Lin et al. 2002). A study of mice with a dominant negative myostatin mutation, which had 23-40% less active myostatin due to a lack of the normal cleavage site, confirmed findings that mice with disrupted myostatin function had more developed skeletal muscles than wild-type littermates. Contrary to the study of McPherron *et al*, 1997, Zhu *et al*, 2000, found that the increase in skeletal muscle mass in mice with a dominant negative myostatin mutation was due to hypertrophy and that hyperplasia was not a significant contributor

(Zhu et al. 2000). Mice are not the only species which exhibit extreme muscle mass when disruption of myostatin function occurs. Several breeds of cattle such as the Belgian Blue and the Piedmontese exhibit double-muscling which causes an increase in muscle mass of about 20-25%. Both of these double-muscled breeds exhibit increased feed efficiency and are able to produce an increased percentage of the most desirable cuts of meat (Kambadur et al. 1997). The myostatin mutation in cattle generally leads to a reduction in size of internal organs, lower viability of offspring, and decreased fertility in females. These difficulties have not been seen in the mice with the myostatin mutation (McPherron et al. 1997).

Myostatin has also been shown to have similar effects in humans. One study which looked at patients after bariatric surgery found that there was a significant reduction of myostatin mRNA expression in muscles of obese subjects after surgery. Although bariatric surgery is accompanied by significant weight loss of both fat free mass and fat mass, after the initial rapid weight loss, body fat reduction continues to decrease but lean body mass does not seem to decrease further(Milan et al. 2004). The decrease in myostatin activity observed after surgery may prevent the further loss of lean body mass (Milan et al. 2004). In a case study involving a child who was born with a spontaneous mutation in the myostatin gene, researchers described the child's extraordinarily muscular thighs and upper arms at birth. At the age of 4½ he continues to have increased muscle mass and size. Ultrasound imaging showed that the cross-section of the quadriceps muscle was 7.2SD above the mean value for children his age, and the thickness of his subcutaneous fat pad was 2.88SD below the mean value. Genetic and serum protein analyses strongly indicate that the child has a loss-of-function mutation in

the myostatin gene. Researchers are continuing to follow the development of this child as he ages (Schuelke et al. 2004).

Myostatin Expression & Fat Accumulation - As mentioned above, myostatin is expressed primarily in the skeletal muscle and to some degree in adipose tissue. Mice with a mutation in the myostatin gene have a significant increase in skeletal muscle mass throughout their body (McPherron et al. 2002). Diabetes mellitus is characterized by insulin resistance and disrupted glucose absorption in skeletal muscle. Since myostatin triggers increased muscle mass and has shown the ability to slow or prevent fat accumulation, it has been a target for possible pharmacological treatment of diabetes (Tsuchida 2004). McPherron and Lee, 2002, showed that loss of myostatin function prevents age-related adipose tissue accumulation and positively affects serum glucose and insulin levels. The myostatin mutation was also able to partially attenuate obese and diabetic phenotypes of two mouse models, Lep ob/ob and KKAy, used to study obesity and diabetes. There are two theories presented in the paper as to how loss of myostatin affects fat deposition and accumulation. One theory is that myostatin has a direct effect on adipose tissue. The second theory is that the myostatin mutation has an indirect effect by causing an increase in skeletal muscle mass therefore utilizing more fuel. The secondary effect is that less energy is available to be stored as fat. The other explanation for an indirect effect of myostatin is that the lack of myostatin in muscle affects the activity of a theoretical second messenger (McPherron et al. 2002).

Myostatin has been shown to have an inhibitory effect on differentiation of pre-adipocytes (Kim et al. 2001). One proposed mechanism for its inhibitory effect is by inhibition of PPAR-γ and CCAAT/enhancer binding proteins (C/EBP), which are key

transcription factors necessary for adipocyte differentiation. Kim *et al*, 2001, demonstrated that treatment of 3T3-L1 (mouse pre-adipocytes) cells with myostatin inhibited adipocyte differentiation in a dose-dependent manner. Expression of PPAR-γ and C/EBP-α in 3T3-L1 cells were significantly reduced when myostatin was added to the cell cultures (Kim et al. 2001). This led to the conclusion that the mechanism by which myostatin inhibited differentiation in pre-adipocytes was by decreasing the expression levels of PPAR-γ and C/EBP-α. However, conflicting reports on the action of myostatin on pre-adipocytes exist. Another study by Artaza *et al*, 2005, demonstrated that recombinant myostatin promoted the differentiation of the multipotent C3H 10T(1/2) cells into adipogenic cells by up-regulating C/EBP-α in a dose-dependent manner (Artaza et al. 2005). In support of this study, Lin *et al*, 2002, reported that myostatin knockout mice had significantly lower fat pad weights, increased muscle mass, and decreased circulating levels of PPAR-γ and C/EBP-α (Lin et al. 2002). Authors theorized that the mechanism by which myostatin knock-out mice decreased fat accumulation was by decreasing adipogenesis which was reflected in the decrease in concentration of adipogenic factors PPAR-γ and C/EBP-α. In conclusion, the mechanism by which myostatin affects adipogenesis and differentiation of pre-adipocytes remains unclear and is still under investigation.

Myostatin Propeptide - The active or mature form of myostatin is generated by cleavage of the precursor protein at the tetrapeptide (RSSR) site. The remaining N-terminal peptide is termed the propeptide (prodomain). The N-terminal propeptide is able to bind the C-terminal and maintain it in a latent dimer *in vitro* and *in vivo* (Wolfman et al. 2003). Other members of the TGF-β superfamily form the biologically active

molecules through cleavage from the precursor protein. In several cases, TGF-β1, -β2, and –β3, the secreted propeptide remains non-covalently bound to the active protein and inactivates it. Thies *et al*, 2001, investigated whether or not the GDF-8 propeptide would be able to bind and inactivate GDF-8. They found that the GDF-8 propeptide was able to complex with GDF-8 when incubated together at a neutral pH, and this greatly reduced the biological activity of GDF-8 mostly through an inhibitory effect on GDF-8 receptor binding (Thies et al. 2001). We and others have demonstrated that over-expression of myostatin propeptide cDNA dramatically enhanced skeletal muscle development, most likely by maintaining myostatin in its latent form (Zhu et al. 2000; Yang et al. 2001; Wolfman et al. 2003; Zhao et al. 2005). The increased muscle mass in the transgenic mice was most likely due to hypertrophy, or increase in muscle cross-sectional size, rather than hyperplasia. Also noted is that the transgenic mice demonstrated much lower epididymal fat pad weights than their wild-type littermates (Yang et al. 2001).

Earlier analyses of transgenic and wild-type mice on a normal-fat or high-fat diet were conducted by our lab (Zhao et al. 2005; Yang et al. 2006). Similar to other studies we found that the transgenic males were heavier than the wild-type animals on the normal diet. We also found that transgenic mice on the high-fat diet showed further enhancement of growth beyond that of the transgenic mice fed a normal-fat diet. Both groups of transgenic mice consumed more kilocalories per day than their wild-type counterparts. Since the majority of the increased weight in transgenic animals was due to increased muscle mass, it indicates that these excess calories were partitioned into muscle mass instead of fat mass. The adipose tissue mass of both transgenic mice was similar to the white adipose tissue mass of the wild-type mice fed the normal-fat diet, but as would

be expected, the wild-type mice on the high-fat diet had much higher fat mass than the other groups. One of the main conclusions of this previous study was that there was a shift of dietary fat utilization toward muscle tissue where dietary fat served as an energy resource for muscle protein synthesis and maintenance, so less energy was stored as fat. This was one of the first studies to show that mice with enhanced muscle mass show minimal fat deposition when they were fed a high-fat diet (Yang et al. 2006).

Prior to the current experiment, transgenic and wild-type animals were weaned at 4 weeks of age and given free access to a normal-fat diet until 9 weeks of age. At this point they were randomly assorted into groups and either fed a normal-fat diet or a high-fat diet with four animals per group. The body weights of the transgenic and wild-type mice were significantly different at 9 weeks of age and at 18 weeks of age; transgenic mice showed increased muscle mass while their wild-type littermates fed a high-fat diet had excessive adipose tissue mass. Muscle triglyceride content of the gastrocnemius muscle from both groups of transgenic mice was significantly lower than those of wild-type mice. The transgenic mice demonstrated similar levels of fat deposition regardless of their dietary fat content, and they had significantly higher daily energy intake than wild-type mice. Fasting blood glucose levels, response to a glucose tolerance test, and insulin sensitivity were similar in transgenic mice fed a high-fat diet, transgenic mice fed a normal-fat diet, and wild-type mice fed a normal diet. The wild-type mice fed a high-fat diet had significantly higher fasting blood glucose levels, abnormal response to the glucose tolerance test, and insulin resistance when challenged with insulin. The concentrations of the metabolic hormones insulin, leptin, and resitin were similar among transgenic mice fed either a normal-fat or high-fat diet. The hormone concentrations in

the transgenic animals were slightly higher but not significantly different from the wild-type mice fed a normal fat diet. The wild-type mice fed a high-fat diet had significantly higher circulating levels of insulin, leptin, and resistin. One anomaly noticed by researchers was that the transgenic mice fed a high-fat diet had much higher levels of serum adiponectin than the wild-type mice and the transgenic mice fed a normal-fat diet. The key findings of this experiment were that the propeptide transgene enhanced muscle growth and also prevented dietary-fat-induced obesity and insulin resistance. Researchers suspect that the enhanced muscling induced by the transgene channeled nutrients into muscle for utilization and affected energy partitioning and storage. Since adiponectin has demonstrated the ability to increase fatty-acid transport and oxidation in skeletal muscle, the increased adiponectin levels may enable the transgenic mice fed a high-fat diet to utilize excess triglycerides in skeletal muscle and prevent accumulation of adipose tissue in fat pads (Zhao et al. 2005).

OBJECTIVES & HYPOTHESIS

Based on the findings of the previous experiment that the transgenic, high-fat diet mice displayed a significant increase in serum levels of adiponectin, as compared to the other three groups of mice, the purpose of this experiment was to determine which fat tissue was expressing the most adiponectin mRNA in each of the four groups. Tissues from the prior experiment were used to determine expression of adiponectin mRNA. We theorized that the combination of the myostatin propeptide transgene and a high-fat diet would demonstrate significant differences in adiponectin mRNA expression among fat depots. Since information on enhancers and inhibitors of adiponectin receptor expression are still under investigation, we also wanted to determine the effects of diet and myostatin

propeptide expression on the mRNA expression levels of the adiponectin receptors (AdipoR1 & AdipoR2) in muscle and liver tissue. We theorized that an increase in adiponectin mRNA expression would correlate to higher mRNA expression levels of AdipoR1 and AdipoR2. PPARs are thought to play a significant role in the expression and actions of adiponectin. We also chose to investigate the expression levels of PPAR-α and PPAR-γ to see if they correlated to the increased serum adiponectin levels. A PPAR response element (PPRE) was discovered in the promoter region of adiponectin, and so we theorized that an increase in adiponectin mRNA expression would correlate to an increase in mRNA expression of PPAR-γ. Since PPAR-α has been thought to increase β-oxidation, we also theorized that the increase in expression of adiponectin mRNA would also increase the expression of PPAR-α mRNA.

CHAPTER 2

CHANGES IN THE mRNA EXPRESSION OF ADIPONECTIN, ADIPONECTIN RECEPTORS, PPAR-α, and PPAR-γ DUE TO DIETARY FAT CONTENT AND MYOSTATIN PROPEPTIDE TRANSGENE EXPRESSION

INTRODUCTION

The expression of myostatin propeptide was able to ameliorate the effects of a high-fat, diet-induced obesity in the transgenic mice. Wild-type mice fed a high-fat diet developed symptoms of obesity and insulin resistance (Zhao et al. 2005). In previous experiments we detected a significant difference in the serum levels of adiponectin. We found that the transgenic, high-fat diet mice had significantly higher levels of circulating adiponectin(Zhao et al. 2005). Degawa-Yamauchi *et al*, 2005, found that expression of adiponectin mRNA negatively correlated with the BMI of individuals and also demonstrated that the serum levels of adiponectin protein were correlated to mRNA expression (Degawa-Yamauchi et al. 2005). In this experiment, we tried to determine which adipose tissue depot was expressing the highest amount of adiponectin mRNA and if there was differential expression of adiponectin, PPAR-α, and PPAR-γ among fat pads from the same animals. Since there is evidence of a PPAR response element (PPRE) in the promoter region of adiponectin (Iwaki et al. 2003), we wanted to determine if the secretion mRNA expression of adiponectin and PPAR-γ were correlated. Also, since Yamauchi *et al*, 2007, demonstrated that the adiponectin receptor AdipoR1 stimulated the expression of PPAR-α (Yamauchi et al. 2007), we wanted to determine if an increase in expression of adiponectin mRNA would also correspond to the increased expression of PPAR-α mRNA in the adipose tissue. We tested the fat tissues from four groups of mice

described below and compared the expression levels of adiponectin, PPAR-α, and PPAR-γ. An increase in the expression of adiponectin would not have significant effects such as improved insulin sensitivity, decreased hepatic glucose production, or increased fatty acid oxidation if the expression of the receptors were not also increased . We also examined the mRNA expression levels of the adiponectin receptors in the gastrocnemius muscle and the liver tissues of these 16 mice to determine if the increase in serum adiponectin was paralleled by an increase in expression of AdipoR1 and AdipoR2 mRNA.

MATERIALS AND METHODS

Animals - Transgenic mice expressing myostatin propeptide were obtained from Dr. Jinzeng Yang (University of Hawai'i, Manoa) and used in this experiment (Yang et al. 2001). Transgenic and wild-type offspring from the propeptide transgenic mice were mated with B6SJL F1. The animals were housed in cages with a constant temperature and 12h light/dark cycle. The animals were weaned at 4 weeks of age and given un-restricted access to a normal fat diet (10% kcal fat, Laboratory Rodent Diet 5001, metabolizable energy 3.04kcal/g, W.F. Fisher & Son, Somerville, NJ) until 9 weeks of age. At this time the male mice were randomly assigned to one of two types of diet (normal fat, 10% kcal fat, Laboratory Rodent Diet 5001; or high fat, 45% kcal fat, Formula D12451, metabolizable energy 4.73 kcal/g, Research Diets) based on genotype (transgenic and wild-type). The mice consumed the respective diets from the age of 9 weeks until the age of 15 weeks. More information on the macronutrient compositions of the diet is available in Appendix 1 (Zhao et al. 2005)

Nine weeks of age in mice is representative of adolescence in humans, and 16-18 weeks of age in mice is representative of adulthood in humans (Zhao et al. 2005). The

mice were given free access to the respective diets and water from weeks 9-15. There were four groups of animals with four animals per group, wild-type/normal-fat diet (WT/NF), transgenic/normal-fat diet (TG/NF), wild-type/high-fat diet (WT/HF), and transgenic/high-fat diet (TG/HF). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Hawai'i.

Tissue Sampling - The mice were killed at 18 weeks of age after an overnight fast. Gastrocnemius, biceps, triceps, longissimus dorsi (LD), and semitendinosus (ST) muscles were dissected and weighed. Liver tissue was dissected and weighed. Subcutaneous, epididymal, mesenteric, and retroperitoneal adipose tissue fat pads were dissected and weighed. The % white adipose tissue (%WAT) mass with respect to the carcass mass given in the results section includes the four fat pads mentioned above. Retroperitoneal fat was not present in all animals and therefore was not used for mRNA expression assays. (Zhao et al. 2005) All tissues were stored at -80°C prior to use.

RNA Extraction and cDNA Preparation - Total RNA was isolated from tissues mentioned above using TRIzol reagent (Invitrogen, Carlsbad, CA) and chloroform. Approximately 0.1g of each tissue was used for the total RNA extraction and prepared on dry ice using forceps and a scalpel. Tissue was then homogenized in TRIzol reagent using a Polytron homogenizer at maximum speed for 60 seconds. In adipose tissue samples, after homogenization and prior to RNA isolation, a layer of insoluble fat was removed as described in the TRIzol protocol. Concentration of total RNA was determined by measuring absorbance at 260 and 280 nm using a Smart Spec 3000 (BioRad, Hercules, CA) Prior to the reverse transcriptase reaction, RNA was treated with 1μL Deoxyribonuclease I (Invitrogen) and the appropriate buffers according to protocol

to remove any residual genomic DNA. cDNA was then synthesized using 2μg total RNA, 1 μL amplification grade SuperScript II Reverse Transcriptase (Invitrogen), and the appropriate reaction buffers to a final volume of 20 μL per reaction tube according to the protocol set by Invitrogen. The cDNA was stored at -80°C until use.

Validation - In order to verify purity of cDNA and specificity of adiponectin, adiponectin receptor 1&2, PPAR-α, PPAR-γ, and GAPDH primers, PCR was conducted using Platinum Taq DNA Polymerase (Invitrogen) and iCycler (BioRad). Amplified cDNA was visualized by using 2% agarose gel electrophoresis, staining with ethidium bromide, and a FluorS Multi-imager (BioRad). (Appendix 2) A standard curve was run using pooled cDNA from mesenteric and retroperitoneal fat to determine adequacy of 0.05μg CDNA sample size for real-time PCR runs (Appendix 3).

Quantitative Real-Time PCR - The relative expression of adiponectin, adiponectin receptor 1, adiponectin receptor 2, PPAR-α, and PPAR-γ were determined, using the appropriate primers (Table 1), by quantitative real-time PCR using the SYBR Green reagent in an ABI 7300 Sequence Detection System (Applied Biosystems, Forrest City, CA). Primers were designed using OligoPerfect Designer (Invitrogen; http://www.invitrogen.com). Each well of the 96-well reaction plate contained a total volume of 50 μL. 0.5 μL of cDNA solution was combined with 1.5μL each of forward and reverse primers (10 pmol/μL), 21.5 μL distilled water, and 25 μL SYBR Green PCR Master Mix (Applied Biosystems). Plates were mixed gently, sealed with optical tape, centrifuged for 10 minutes at 2000 rpm, and placed in the 7300 Real-Time PCR system (Applied Biosystems). Optimal annealing temperatures for the primers used was determined to be 60°C and 45 cycles were run. The abundance of each mRNA transcript

was measured and expressed in comparison to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which is a steady state enzyme which catalyzes the sixth step of glycolysis. Relative expression of mRNA was determined using calculations based on threshold cycle (Ct) according to protocol set by Applied Biosystems and were expressed as fold change compared to wild-type/normal-fat diet mice. Fold change was calculated by subtracting the Ct number of the gene of interest from the Ct of the endogenous control gene, in this case GAPDH. The result of this calculation was termed ΔCt. The ΔCt values for adiponectin, PPAR-α, and PPAR-γ are presented in Appendix 4. The ΔCt values for the adiponectin receptors AdipoR1 and AdipoR2 are presented in Appendix 5. The ΔCt of the treatment group was subtracted from the ΔCt of the control group, in this case the WT/NF mice. The result of this was termed ΔΔCt. The fold change was calculated as $2^{-\Delta\Delta Ct}$ (Livak et al. 2001).

Statistical Analysis - The data for mean comparisons were analyzed by using the JMP program. (SAS Inst., Cary, NC). Significant differences between the mass of the main muscles and adipose tissue mass were analyzed by ANOVA and Student's t-test. The effects of genotype, diet, and fat pad location on the mRNA expression levels of adiponectin, adiponectin receptors, PPAR-α, and PPAR-γ were analyzed by ANOVA, two-tailed Student's t-test, and Tukey's HSD test. Least square means and their standard errors are reported. Significance was determined at $p < 0.05$.

RESULTS

Carcass weights, muscle mass, and fat pad mass - The two groups of transgenic mice, normal-fat and high-fat diet, displayed the largest carcass weights of the four groups, 16.23±1.09g and 15.72±0.36g, respectively. Carcass weights for the two

transgenic groups were significantly higher than the carcass weights of the two wild-type groups. Mean carcass weight for the wild-type/normal-fat (WT/NF) diet group was 10.68±0.28g and 12.13±0.32g for the wild-type/high-fat diet (WT/HF) group. The transgenic/normal-fat (TG/NF) mice had significantly heavier muscle masses (gastrocnemius, biceps, triceps, LD, and ST) than the other three groups of animals. The transgenic/high-fat (TG/HF) diet group had significantly heavier gastrocnemius, biceps, triceps, and LD muscles than both wild-type groups. The WT/HF group had heavier biceps and triceps than the WT/NF group. There was no significant difference among the wild-type animals with respect to the gastrocnemius, LD, or ST muscles. The value of WAT as percent of carcass mass for the WT/HF group was 25.96±2.20% which was significantly higher than the other three groups of mice. The mass of the subcutaneous, epididymal, and retroperitoneal pads in the WT/HF group were significantly higher than the TG/HF group. Muscle and fat pad weights are presented in Table 2.

Adiponectin mRNA Expression in Adipose Tissues - The mRNA expression level of adiponectin was determined by using the ΔCt values to calculate fold change using the subcutaneous fat of the WT/NF mice as the reference tissue. The adiponectin expression level was the highest in the epididymal fat of the TG/HF group of mice with a value of 3.10±0.70-fold. The adiponectin expression level of the TG/HF group in epididymal fat was significantly higher than the expression level in the three fat pads of all other groups in this experiment except for the epididymal fat of the TG/NF group which was 2.07±0.15-fold. Within the epididymal fat pad, the WT/HF group had the lowest expression of adiponectin at 0.25±0.09-fold. There were no significant differences in adiponectin expression between groups noted in the subcutaneous and

mesenteric fat pads. Statistical analysis showed that the location of the fat pads and the genotype of the animals had significant effects on the expression of adiponectin. There was also evidence of an interaction between the genotype and fat pad location which increased adiponectin expression. Although there was an interaction among all three factors to produce effects on levels of adiponectin expression there was no indication that diet alone had any effect. Fold change results are presented in Table 3 and Figure 2.

PPAR-α mRNA Expression in Adipose Tissues - The epididymal fat of the TG/HF group expressed the highest levels of PPAR-α mRNA with an expression level of 8.16±0.81-fold higher than the reference group (WT/NF, subcutaneous fat). There was no significant difference in PPAR-α expression within the subcutaneous fat, and the expression levels in this tissue were lower than expression levels in the other two tissues, epididymal and mesenteric fat. The WT/HF group expressed the lowest levels of PPAR-α within both the epididymal and mesenteric fat pads, at levels of 1.74±0.72-fold and 1.31±0.54-fold respectively. Statistical analysis showed that the location of the fat pad, the genotype of the animals, and the fat content of the diet had significant effects on the expression of PPAR-α mRNA. There were also significant effects shown on mRNA expression by interactions between fat pad location and genotype, genotype and diet, and fat pad location and diet. All three factors played a role in the differences in levels of PPAR-α mRNA expression between the four groups of animals and the locations of the adipose tissue stores. Fold change results are presented in Table 3 and Figure 3

PPAR-γ mRNA Expression in Adipose Tissues - The expression level of PPAR-γ mRNA was highest in the epididymal fat of the TG/HF group at 2.56±0.26-fold compared to the reference group (WT/NF, subcutaneous fat). When all three fat pads

were compared, the level of PPAR-γ mRNA expression by the epididymal fat of the TG/HF group was significantly higher than the level of the expression by the subcutaneous fat of the TG/NF and WT/HF group, the epididymal fat of the WT/HF group, and the mesenteric fat of the TG/NF and TG/HF groups by approximately 2-fold. With respect to the mesenteric and subcutaneous fat pads, there was no significant difference in expression of PPAR-γ mRNA. Statistical analysis showed the location of the fat pad alone had significant effects on PPAR-γ mRNA expression. There were significant effects of the interaction between fat pad location and genotype, and genotype and diet. Fold change results are presented in Table 3 and Figure 4.

Adiponectin Receptor 1 & 2 mRNA Expression in Muscle and Liver Tissue - There were no significant differences in adiponectin receptor mRNA expression in the muscle tissue of all four groups, but according to statistical analysis there was an effect of diet on the expression of AdipoR2 in the muscle. In the liver, the expression of AdipoR1 was highest in the WT/NF group. The expression of AdipoR1 mRNA in the WT/NF group was significantly higher than the expression levels of the other three groups by approximately 1-fold. Statistical analysis demonstrated that the effect of genotype and diet on the expression of AdipoR1 in the liver was significant. The interaction between diet and genotype also had a significant interaction on the expression of AdipoR1 mRNA. The two wild-type groups also had significantly higher levels of AdipoR2 expression in the liver, but only by about 0.5-fold above the transgenic groups. Statistical analysis showed a significant effect of genotype and diet separately as well as an interaction between the genotype and diet on the expression of PPAR-γ mRNA. Fold change results are presented in Table 4 and Figure 5.

DISCUSSION

In the previous experiment by Yang and Zhao, the transgenic, high-fat diet mice did not display obesity which was seen in the wild-type mice fed the high-fat diet. The transgenic mice fed the high-fat diet had a phenotype similar to the transgenic, normal-fat diet mice (Yang et al. 2006). The two groups of transgenic mice (TG/NF and TG/HF) had significantly higher carcass masses than the wild-type groups. The TG/NF mice had significantly heavier muscles than the TG/HF mice, but both transgenic groups had significantly heavier individual muscles than the wild-type groups. In the study by Zhao *et al*, 2005, the consumption of a high-fat diet led to obesity, high fasting glucose levels, and insulin resistance in the WT/HF group, but the transgenic mice fed the high-fat diet did not become obese or display symptoms of insulin resistance or obesity (Zhao et al. 2005). In a smaller subset of animals from the previous experiment we also found that the WT/HF mice had an obese phenotype, while the TG/HF mice did not. The % WAT (white adipose tissue) compared to the carcass mass of the WT/HF group was significantly higher than the percentage of WAT in the other three groups. The mass of the individual fat pad weights of the WT/HF group were also significantly higher than the fat pad weights of the other three groups. A previous experiment using the same subjects demonstrated that the TG/HF mice had significantly higher serum adiponectin concentrations than the other groups (Zhao et al. 2005). The increase in serum adiponectin may be one mechanism by which the TG/HF mice were able to prevent increased fat accumulation that occurred in the WT/HF group. The expression of the myostatin propeptide transgene may have increased the skeletal muscle accumulation of the TG/HF mice above the wild-type animals and this required more energy for

maintenance and growth. The increase in energy utilization by skeletal muscle in the transgenic mice may have used the energy provided by the high-fat diet and prevented the storage of energy in adipose tissue seen in the WT/HF mice. Since adiponectin has been found to increase beta-oxidation (Tomas et al. 2002); the increase in serum adiponectin seen in the TG/HF group may have occurred in response to an increased need for energy by the skeletal muscles for growth and maintenance.

Adiponectin has been associated with many beneficial properties both in humans and in animal models. Due to the positive associations of adiponectin with improved insulin sensitivity and glucose tolerance and increased fatty acid oxidation, researchers have been concentrating on determining the factors which may induce expression of adiponectin and adiponectin receptors (Tomas et al. 2002; Yamauchi et al. 2002). The role of adipocytes in the development of chronic diseases such as diabetes, cardiovascular disease, and hypertension has not been completely elucidated. Expression of many adipocytokines increases with obesity because the total fat mass and number of adipocytes has increased. Adiponectin is one of the few adipocytokines whose levels have been shown to decrease with obesity (Hu et al. 1996). The mechanism by which obesity decreases adiponectin levels is still unclear. Researchers have shown that different adipose tissue fat pads express genes at varying levels, meaning that certain genes are more actively transcribed than others (Okuno et al. 1998). Fisher *et al*, 2002, found that expression of adiponectin mRNA and protein secretion was different in distinct fat depots and much lower in human diabetic subjects and those with insulin resistance (Fisher et al. 2002). We found that the obese WT/HF mice expressed the

lowest levels of adiponectin in epididymal fat which correlates to the findings of Fisher *et al.*

We also demonstrated adipose tissue-specific variation in expression of adiponectin in the same mice. The epididymal fat pad appears to be the most active with respect to mRNA expression of adiponectin. This supports the conclusion that all adipose tissue is not the same; fat pads in different anatomical locations may have different functions and play different roles in maintaining energy homeostasis (Guo et al. 2004). Our findings correlate with this because the three fat pads we analyzed had different patterns of expression for adiponectin. Transgenic mice fed a high-fat diet showed the highest expression of adiponectin mRNA in epididymal fat and the expression level was significantly greater than adiponectin mRNA expression in the two wild-type groups (WT/NF and WT/HF). When the adiponectin mRNA expression levels of the three fat pads were examined we found that the epididymal fat was secreting the highest levels of adiponectin mRNA of the three fat pads. Statistical analysis showed that the location of the fat pad had a significant effect on the expression of adiponectin mRNA. There was no significant difference in adiponectin mRNA expression among the subcutaneous and mesenteric fat pads; leading us to believe that the variations seen in serum adiponectin is not likely due to differential mRNA expression in either the mesenteric or the subcutaneous fat tissue. Fisher *et al*, 2002, and Degawa-Yamauchi *et al*, 2005 both demonstrated that the expression of adiponectin mRNA strongly correlated to protein expression and serum concentration of adiponectin (Fisher et al. 2002; Degawa-Yamauchi et al. 2005). Comparison of the three fat pads demonstrated that the fad pad that is most likely leading to the increase in serum adiponectin observed in the

previous experiment is the epididymal fat since we found variations in expression among the four groups with the TG/HF group having the highest expression level. The increase in adiponectin mRNA expression correlates to the increased serum adiponectin found in the previous experiment by Zhao et al, 2005 (Zhao et al. 2005).

Abdominal fat pads are known to be particularly metabolically active and tend to have the greatest effect on the liver and other visceral organs because of its close proximity to them, and also because the secretions are released directly into the hepatic portal vein which can directly influence metabolic processes in the liver (Matsuzawa et al. 1995; Funahashi et al. 1999; Kershaw et al. 2004). The epdidymal and mesenteric fat pads are located within the abdominal cavity and adipocytokines released from these tissues should have a greater effect in the liver than factors released from the subcutaneous fat. In the case of adiponectin, secretion into the hepatic portal vein by visceral fat tissue would have a beneficial effect because of its ability to decrease hepatic gluconeogenesis and increase fatty acid oxidation (Berg et al. 2001; Combs et al. 2001; Yamauchi et al. 2002). Another possible explanation for the increase in adiponectin mRNA expression of epididymal fat of the TG/HF mice is the size of the adipocytes in the epididymal fat of the TG/HF mice. Several studies have shown that smaller adipocytes are more metabolically active (Okuno et al. 1998; Kadowaki et al. 2003). If the size of the adipocytes in the epididymal fat of the TG/HF mice is smaller than the other three groups this could explain the difference in adiponectin mRNA expression we observed. Although we did not have data on size of adipocytes in the four groups of animals it is something we could investigate in future studies.

Activation of PPAR-α induces the expression of fatty acid transport proteins and key enzymes involved in beta-oxidation such as FATP/CD36 and acyl-CoA oxidase. PPAR-α also induces glucose sparing possibly by directly inducing the expression of pyruvate dehydrogenase kinase 4 or indirectly by synthesizing ketone bodies and increasing fatty acid oxidation (Ferre 2004). Yamauchi *et al*, 2007, reported that AdipoR2 activation increased the expression of the gene encoding PPAR-α while AdipoR1 activation had no effect on these genes(Yamauchi et al. 2007). If AdipoR2 is able to increase activity of PPAR-α, then theoretically an increase in expression of adiponectin should also lead to an increase in PPAR-α expression. We found that the epididymal fat of the TG/HF mice was expressing the highest level of PPAR-α mRNA. This correlated to the increased expression of adiponectin mRNA found in the epididymal fat of these mice. Since adiponectin is thought to enhance expression of PPAR-α, it makes sense that the tissue of the TG/HF which expressed the most adiponectin mRNA would also express the most PPAR-α mRNA. Two possibilities exist to explain the increase in expression of PPAR-α mRNA in the epididymal fat of the TG/HF mice. One is that the increase in fat-content of the diet led to increased serum levels of linoleic acid, a PPAR-α ligand, that triggered the increase in expression of PPAR-α mRNA. Although this may have happened to some extent, if this were the only reason PPAR-α expression increased then the WT/HF group would also have been expected to have increased PPAR-α mRNA expression, which was not the case. Another argument against the increased fat-content of the diet leading to the increase in PPAR-α mRNA expression is that fatty acids are low-affinity PPAR-α ligands. More likely is the second possibility that the increased serum concentration of adiponectin and the

increased expression of adiponectin mRNA stimulated the activation of PPAR-α by binding to AdipoR2 and led to the increased PPAR-α mRNA expression which was observed in the epididymal fat of the TG/HF mice.

One of the primary functions of PPAR-γ is to induce pre-adipocytes to differentiate into adipocytes (Ferre 2004). This function can explain the increase in PPAR-γ expression in the animals fed the high-fat diet. Animals fed a high-fat diet are more likely to store excess energy in the adipocytes because of the increase in serum concentrations of fatty acids and increased energy content of the diet. Adipose tissue mass results from either an increase in the volume of the existing adipocytes, hypertrophy, or from an increase in the number of adipocytes, hyperplasia (Kokta et al. 2004). If the adipocytes are not able to increase in volume any more, new adipocytes are needed and PPAR-γ is one of the signal molecules which mediate their conversion from pre-adipocytes. The high-fat diet mice had higher circulating free fatty acids, which are PPAR-γ ligands, and in order to clear these free fatty acids it would be necessary to increase differentiation of pre-adipocytes. This is reflected in the increase in expression of PPAR-γ seen in most tissues of the high-fat diet mice. It is possible that the increase in dietary fat content led to increased circulating concentrations of fatty acids which bound to and activated PPAR-γ. Kim et al, 2001, reported that myostatin is able to inhibit adipocyte differentiation and inhibit PPAR-γ (Kim et al. 2001). This leads to another possibility for the increase in PPAR-γ mRNA expression. Possibly, the myostatin propeptide inactivated myostatin and consequently decreased any inhibitory effect myostatin may have had on PPAR-γ expression.

In this experiment we found that there was a correlation between the expression of adiponectin and the expression of PPAR-γ. We found PPAR-γ expression levels were significantly higher in the epididymal fat of TG/HF mice than the WT/HF mice. This corresponds with the finding of significantly higher expression level of adiponectin in the epididymal fat of the TG/HF mice than the WT/HF mice. One possible explanation for the increase in PPAR-γ expression in the transgenic, but not the wild-type mice fed the high-fat diet is that the adipocyte size in the fat pads of the WT/HF mice were larger than the adipocytes of the TG/HF mice leading to less secretion or activity of the adipocytokines in this group. The results of our study strengthen reports that PPAR-γ agonists may improve insulin sensitivity by activating adiponectin. Also, it strengthens the evidence that supports the presence of a PPRE in the promoter region of adiponectin (Iwaki et al. 2003). The increased levels of PPAR-γ mRNA expression observed in the epididymal fat of TG/HF mice could be due to the interaction between myostatin propeptide and dietary fat content. The expression of the myostatin propeptide led to increased muscle mass which needed more energy for growth and maintenance of skeletal muscle. The increased concentrations of fatty acids in the serum led to activation of PPAR-γ which led to the binding and activation of the PPRE in the promoter region of adiponectin causing the increase in adiponectin mRNA expression in the epididymal fat pad. The high-fat diet may induce the expression of PPAR-γ because of the increase in circulating free fatty acids and triglycerides which are ligands of PPARs. This increase in concentration of circulating fatty acids and triglycerides may create the need to store these energy-rich molecules making the conversion of pre-adipocytes to adipocytes necessary. PPAR-γ is required for the initiation of conversion of adipocytes and a

secondary result of an increase in PPAR-γ may be increased adiponectin expression because of the PPRE present in its promoter region. Since PPAR-γ agonists are able to stimulate expression of adiponectin, it seems that the increase in adiponectin mRNA expression and protein secretion is preceded by and induced by the increase in PPAR-γ expression (Maeda et al. 2001; Phillips et al. 2003). Whether the increase in PPAR-γ expression is due to the presence of increased fatty acid ligands or the decrease in inhibition by myostatin, this experiment indicates that one possible mechanism by which PPAR-γ may be able to ameliorate insulin resistance is by increasing adiponectin expression that leads to increases in fatty acid oxidation, increased glucose uptake, and decreased hepatic gluconeogenesis.

There was a significant effect of diet on the expression of the adiponectin receptors, AdipoR1 and AdipoR2. Other studies have found the expression of adiponectin receptors to be reduced in animals which are obese, hyperinsulinemic, or hyperglycemic (Fang et al. 2005; Inukai et al. 2005). Increased concentrations of plasma FFAs and triglycerides along with accumulation of triglycerides and long-chain acyl-CoAs in muscle and liver tissue are associated with increased insulin resistance and obesity (Roden et al. 1996; Griffin et al. 1999; Boden et al. 2001; Hulver et al. 2003; Cahova et al. 2006). Chronic intake of a high-fat diet would lead to increased circulation of FFAs and thus a high-fat diet could correspond with decreased adiponectin receptor expression. Our results showed that a high-fat diet independently decreased the expression of AdipoR1 and AdipoR2 in liver and decreased expression of AdipoR2 in muscle. One possibility for the decreased insulin sensitivity in individuals on a high-fat diet is the decrease in expression of adiponectin receptors related to increased triglyceride

and long-chain fatty-acyl-CoAs in circulation and accumulating in these muscles. Chronic high-fat diet has been associated with the development of obesity and insulin-resistant states. Adiponectin levels are inversely correlated with obesity and insulin resistance, and if adiponectin receptor expression is also decreased in these states, this could lead to perpetuation of the obesity cycle (Arita et al. 1999). The decrease in both adiponectin and adiponectin receptor expression in obese and/or insulin resistant states would promote a decrease in fatty acid oxidation leading to further accumulation of triglycerides and free fatty acids in muscle leading to a further decline in insulin sensitivity. Also, a decrease in activity of adiponectin and adiponectin receptors would be associated with decreased glucose uptake and increased hepatic gluconeogenesis. These actions would further increase hyperglycemia and hyperinsulinemia, further pushing individuals into an insulin-resistant state.

LIMITATIONS

One of the most significant limitations of this experiment is the small sample size of animals used. Each group of animals, wild-type/normal-fat diet, wild-type/high-fat diet, transgenic/normal-fat diet, transgenic/high-fat diet, only had 4 subjects, and in most cases there was an outlier which was removed for statistical analysis. Consequently, for most of the genes examined it was difficult to elicit statistically significant results. Another limitation was that in some cases the RNA extraction from tissue consumed all the fat tissue which was removed from the individual animals, and the tissue could not be used for other analyses. Along the same lines, the fact that the tissue was frozen prevented antibody-staining analysis to back up results gathered from real-time PCR analysis. Another limitation of the use of frozen tissues was that we were not able to

examine the size of the adipocytes to determine if they were different between the four groups of animals.

TABLE 1. Primer sequences for adiponectin, adiponectin receptor 1, adiponectin receptor 2, PPAR-α, PPAR-γ, and GAPDH

Gene name (murine)	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)
Adiponectin	ggaacttgtgcaggttggat	cgaatgggtacattgggaac	273
Adiponectin Receptor 1	acgttggagagtcatcccgtat	agtgtggaagagccaggaga	287
Adiponectin Receptor 2	ggatgtggaagtcgtgtgtg	acctggtcaaacgagacacc	230
PPAR-α	ctccagacgaaccatccagat	tgtacgagctgcgcatgct	101
PPAR-γ	atgccaaaaatatccctggtttc	ggaggccagcatcgtgtaga	100
GAPDH	acccagaagactgtggatgg	cacattgggggtaggaacac	171

TABLE 2. Mass of carcass, main muscles, and fat pads.

	NORMAL-FAT DIET		HIGH-FAT DIET	
	WILD-TYPE	TRANSGENIC	WILD-TYPE	TRANSGENIC
Carcass	10.68±0.28 b	16.23±1.09 a	12.13±0.32 b	15.72±0.36 a
Gastrocnemius	0.13±0.02 c	0.44±0.02 a	0.16±0.02 c	0.27±0.02 b
Biceps femoris	0.16±0.01 d	0.50±0.01 a	0.24±0.02 c	0.31±0.02 b
Triceps brachii	0.13±0.02 d	0.62±0.02 a	0.22±0.02 c	0.34±0.02 b
Longissimus dorsi	0.38±0.03 c	0.96±0.12 a	0.55±0.05 b,c	0.64±0.06 b
Semitendinosus	0.22±0.04 b	0.47±0.01 a	0.20±0.04 b	0.20±0.01 b
Subcutaneous fat	0.42±0.05 b	0.52±0.07 b	1.12±0.04 a	0.42±0.13 b
Epididymal fat	0.44±0.07 b	0.67±0.11 b	1.11±0.11 a	0.51±0.10 b
Mesenteric fat	0.21±0.05 b	0.45±0.04 a	0.39±0.03 a,b	0.22±0.04 b
Retroperitoneal fat	0.04±0.02 b	0.24±0.08 a,b	0.52±0.06 a	0.21±0.08 b
WAT % carcass wt	10.39±1.18 b	11.73±1.01 b	25.96±2.20 a	8.61±2.23 b

Muscle and fat pad weights and % white adipose tissue (WAT) are expressed as means ± SEM. Within each row, levels not connected by the same letter are significantly different.

TABLE 3. Fold change results for Adiponectin, PPAR-α, and PPAR-γ in adipose tissues.

	SUBCUTANEOUS				EPIDIDYMAL				MESENTERIC			
	NORMAL FAT		HIGH-FAT		NORMAL FAT		HIGH-FAT		NORMAL FAT		HIGH-FAT	
	WT	TG	WT	TG	WT	TG	WT	TG	WT	TG	WT	TG
Adiponectin	1.10±0.12 b,c	0.41±0.38 c	1.27±0.56 b,c	1.08±0.15 b,c	0.87±0.34 b,c	2.07±0.15 a,b	0.25±0.09 c	3.10±0.70 a	0.14±0.01 c	0.37±0.03 c	0.74±0.23 b,c	0.11±0.02 c
PPAR-α	1.01±0.55 c	1.03±0.49 c	1.02±0.36 c	1.51±0.70 c	4.64±0.60 a,b,c	6.08±0.98 a,b	1.74±0.72 c	8.16±0.81 a	5.60±0.33 a,b	4.18±0.45 b,c	1.31±0.54 c	3.56±1.50 b,c
PPAR-γ	1.68±1.17 a,b,c	0.69±0.47 b,c	0.40±0.13 c	1.49±0.38 a,b,c	1.46±0.19 a,b,c	1.91±0.31 a,b	0.55±0.23 b,c	2.56±0.26 a	1.42±0.22 a,b,c	0.27±0.22 c	1.68±0.17 a,b,c	0.74±0.23 b,c

WT=Wild-type, TG=Transgenic. Data is presented as means ± SEM. Within the same row, levels not connected by the same letter are significantly different. Fold change was calculated as 2-ΔΔCt and quantified by Real-Time PCR.

	Statisctical Significance						
	FP	GEN	DIET	FP*GEN	GEN*DT	FP*DT	FP*GEN*DT
Adiponectin	****	*		****			*
PPAR-α	****	***	*	**	**	*	
PPAR-γ	**			****	**		

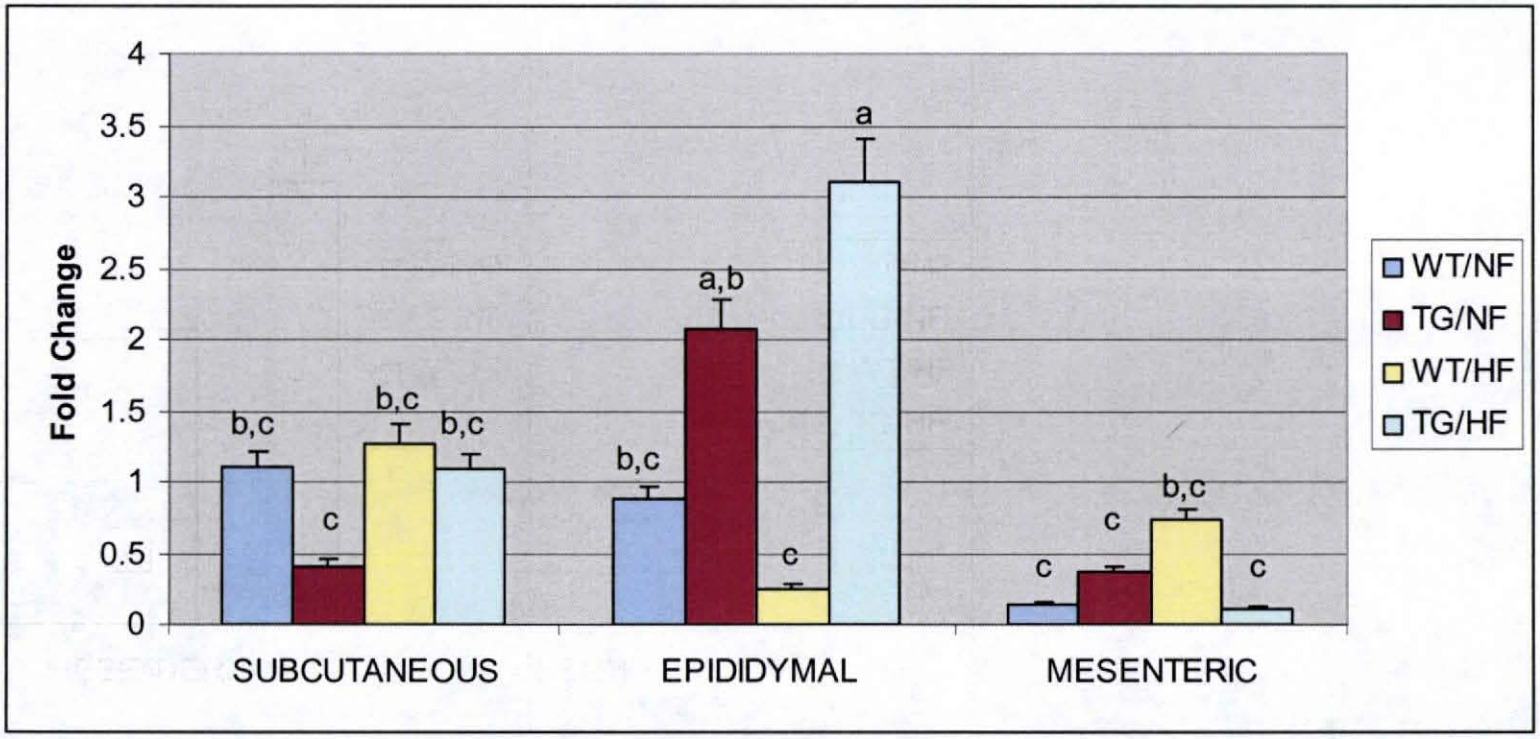
FP = fat pad location, GEN = genotype, DT = diet.
Significance was determined at * p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001

TABLE 4. Fold change results for AdipoR1 and AdipoR1 in gastrocnemius muscle and liver tissue.

		NORMAL FAT		HIGH FAT		SIGNIFICANCE		
		Wild-Type	Transgenic	Wild-type	Transgenic	GENOTYPE	DIET	GENOTYPE*DIET
MUSCLE	AdipoR1	1.53 a (0.18-2.88)	2.62 a (2.14-2.85)	1.25 a (1.02-1.48)	0.15 a (0.14-0.16)			
	AdipoR2	1.16 a (0.57-1.75)	1.48 a (2.15-3.11)	0.59 a (1.02-1.48)	0.27 a (0.14-0.16)		*	
LIVER	AdipoR1	1.03 a (0.85-1.21)	0.11 b (0.07-0.15)	0.08 b (0.06-0.10)	0.46 b (0.33-0.59)	*	*	***
	AdipoR2	1.32 a (1.29-1.35)	0.71 b (0.70-0.72)	1.18 a (1.05-1.31)	0.50 b (0.49-0.51)	****	*	**

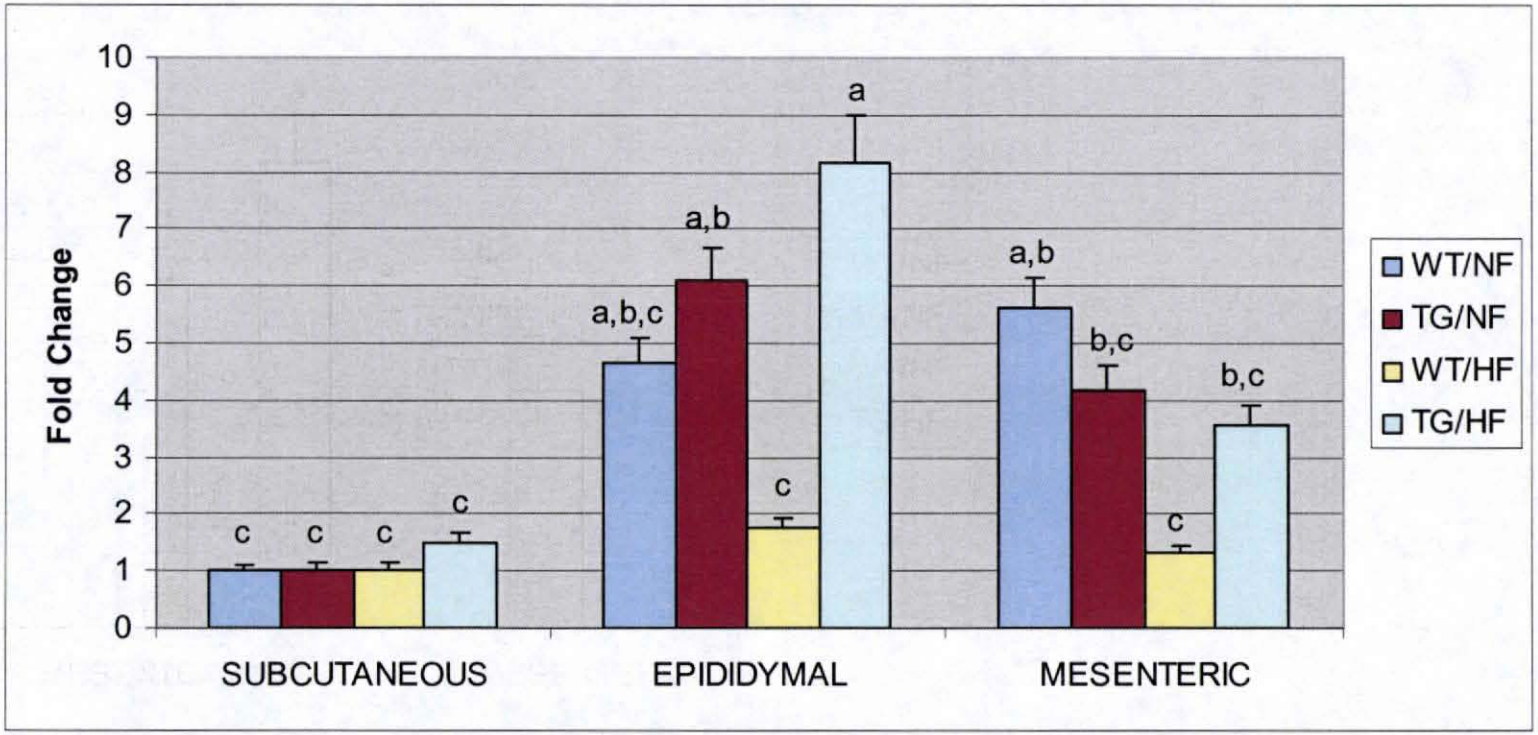
Data is presented as means (ranges). Muscle tissue sampled was gastrocnemius muscle. Within the same row, levels not connected by the same letter are significantly different. Fold change was calculated as $2^{-\Delta\Delta Ct}$ by Real-Time PCR. Significance was determined at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

FIGURE 2. Expression of adiponectin mRNA as fold change in subcutaneous, epididymal, and mesenteric fat.



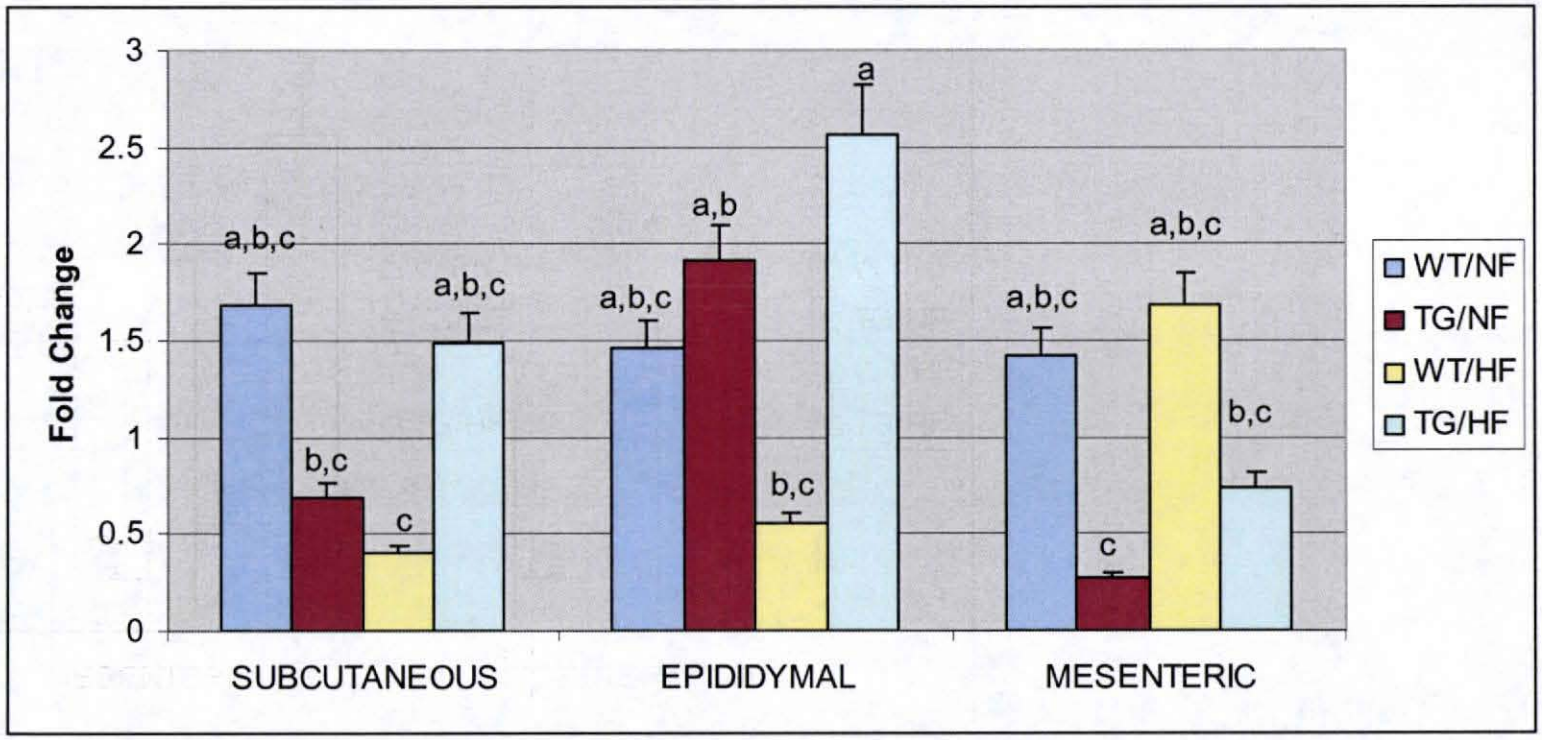
Data is expressed as mean ± SEM. Levels not connected by the same letter are significantly different.
Fold change was calculated as $2^{-\Delta\Delta Ct}$ and was quantified by real-time PCR.

FIGURE 3. Expression of PPAR-α mRNA expression as fold change in subcutaneous, epididymal, and mesenteric fat.



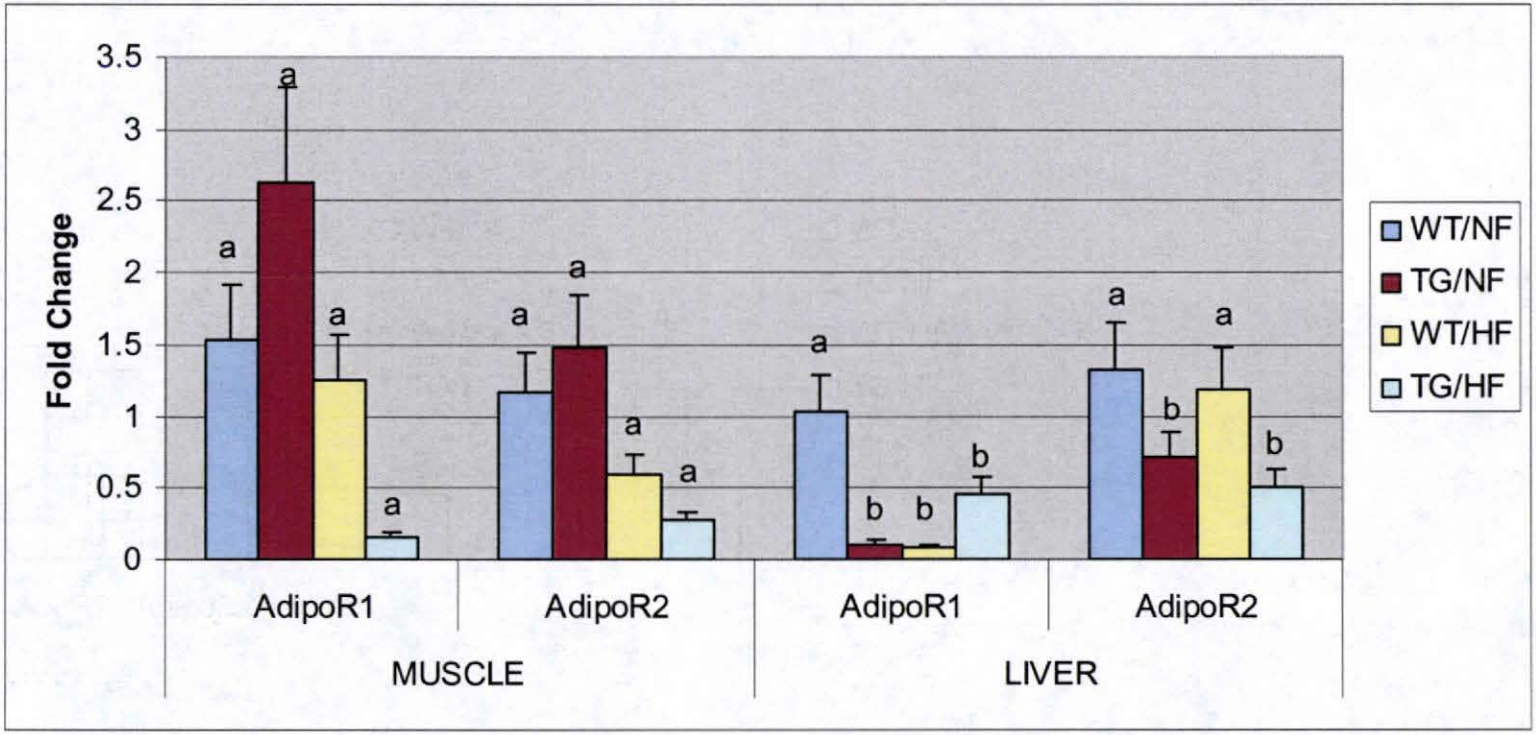
Data is expressed as mean ± SEM. Levels not connected by the same letter are significantly different.
Fold change was calculated as $2^{-\Delta\Delta Ct}$ and was quantified by real-time PCR.

FIGURE 4. Expression of PPAR-γ mRNA as fold change in subcutaneous, epididymal, and mesenteric fat.



Data is expressed as mean ± SEM. Levels not connected by the same letter are significantly different.
Fold change was calculated as $2^{-\Delta\Delta Ct}$ and was quantified by real-time PCR.

FIGURE 5. Expression of adiponectin receptor 1 & 2 mRNA in muscle & liver tissue as fold change.



Data is expressed as mean ± SEM. Within each group, levels not connected by the same letter are significantly different. Fold change was calculated as $2^{-\Delta\Delta Ct}$ and was quantified by real-time PCR.

CHAPTER 3

CONCLUSIONS & SIGNIFICANCE

The potential for adiponectin and adiponectin receptors to be targets for drugs which would treat diabetes, metabolic syndrome, coronary artery disease, and stroke is great. The current generation is more greatly affected by obesity and chronic diseases associated with obesity than previous generations. A combination of increased caloric density, food availability, and decreased energy expenditure in the general population has pushed the energy balance scale towards the accumulation of energy in adipose tissues. Adiponectin appears to have the potential to increase energy expenditure through increased fatty acid oxidation, decrease adhesion of leukocytes associated with cardiovascular disease, decrease the amount of triglycerides stored within muscle tissue which improves insulin sensitivity, and decrease hepatic glucose production (Ouchi et al. 1999; Yamauchi et al. 2002; Cahova et al. 2006). These factors make adiponectin a possible target for treatment of diabetes and insulin resistance. PPAR-γ agonists such as TZDs have been shown to increase circulating adiponectin concentrations and also improve insulin sensitivity among subjects with diabetes. Diabetes increases risk for cardiovascular disease, and both CVD and diabetes are associated with low circulating plasma adiponectin levels especially in those who have both conditions (Funahashi et al. 1999; Ouchi et al. 1999).

Our research demonstrates that there is significant interaction between skeletal muscle tissue and adipose tissue depots which can lead to amelioration of insulin resistance resulting from chronic high-fat feeding. The interactions between the myostatin propeptide transgene and high-fat feeding led to an increase in expression of

adiponectin in one of the larger abdominal adipose tissue accumulation sites, the epididymal fat pad. The increase in adiponectin expression of transgenic mice on the high fat diet was accompanied by increased muscle mass similar to transgenic mice on a normal-fat diet and accumulation of fat similar to wild-type animals on a normal-fat diet. The wild-type mice on a high-fat diet showed increased insulin resistance and decreased serum adiponectin in a previous experiment (Zhao et al. 2005). Adipose tissue from the same animals in the prior experiment by Yang and Zhao which showed high serum adiponectin in TG/HF mice was used for the current experiment. From the current experiment we conclude that the increased expression of adiponectin mRNA seen in the epididymal fat pad of the TG/HF mice is most likely responsible for the increased serum adiponectin observed in the animals in the previous experiment. The findings of differential secretion of adiponectin by specific fat pads is supported by studies which also showed that different fat pads secrete and express adipocytokines to varying degrees and that the size of the adipocytes in the fat pads may play a role in this differing expression (Zhang et al. 2002; Guo et al. 2004).

We were able to demonstrate increased mRNA expression of both PPAR-α and PPAR-γ in the epididymal fat of the TG/HF mice which showed increased adiponectin mRNA expression. The correlation observed between adiponectin and PPAR-γ mRNA levels is most likely related to the presence of the PPRE in the promoter region of adiponectin. We found that animals which showed increased levels of PPAR-γ mRNA expression also demonstrated increased levels of adiponectin expression. This leads to the conclusion that some of the effects PPAR-γ agonists have on improvement of insulin

sensitivity may be partially due to the increase in adiponectin expression and the subsequent positive actions of adiponectin.

The decrease in adiponectin receptors seen in the high-fat diet group is disturbing because even if circulating adiponectin levels did not decrease, the lower number of receptors on the membranes available to bind adiponectin would decrease the possible positive actions seen if adiponectin was administered exogenously. It may not be sufficient to increase circulating concentrations of adiponectin, but it may be necessary to find ways to stimulate expression of adiponectin receptors in order to increase fatty acid oxidation, increase insulin sensitivity, and decrease hepatic gluconeogenesis.

Finally, as far as we know, we are the first group to demonstrate the interaction between fat content of the diet and the effects of the myostatin propeptide transgene. Muscle tissue is an important location for the utilization of energy, and the transgene expression affects the partitioning of energy towards the muscle tissue allowing a significantly higher proportion of total energy expenditure to go towards development and maintenance of skeletal muscle. If we can increase the skeletal muscle mass in obesity or insulin resistant states, we may be able to mimic the partitioning of energy away from storage in adipose tissues and towards building and maintenance of lean body mass.

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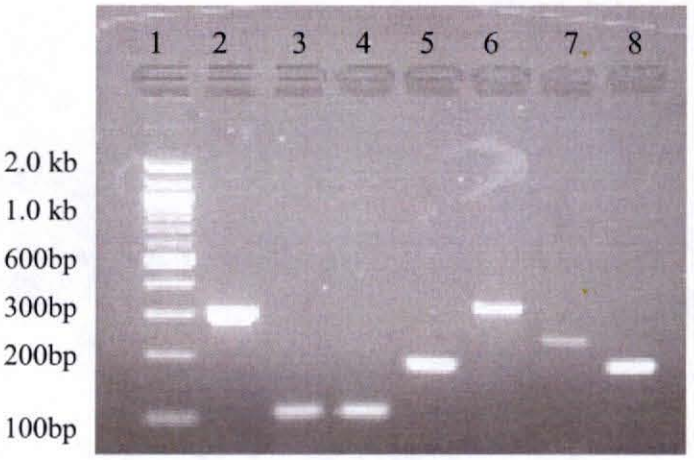
APPENDIX 1. Feed composition analysis

	Laboratory Rodent Diet 5001		Research Diet #D12451	
	gm%	kcal%	gm%	kcal%
Protein	23	28	24	20
Carbohydrate	72.5	60	41	35
Fat	4.5	12	24	45
Total		100		100
kcal/g	3.04		4.73	

Nutritional analysis for Laboratory Rodent Diet 5001 (normal-fat diet) obtained from W.F. Fisher & Son, ingredient list available at http://www.labdiet.com/5001.htm. Nutritional analysis for Research Diet # D12451 (high-fat diet) obtained from Research Diets Inc, ingredient list available at http://www.researchdiets.com.

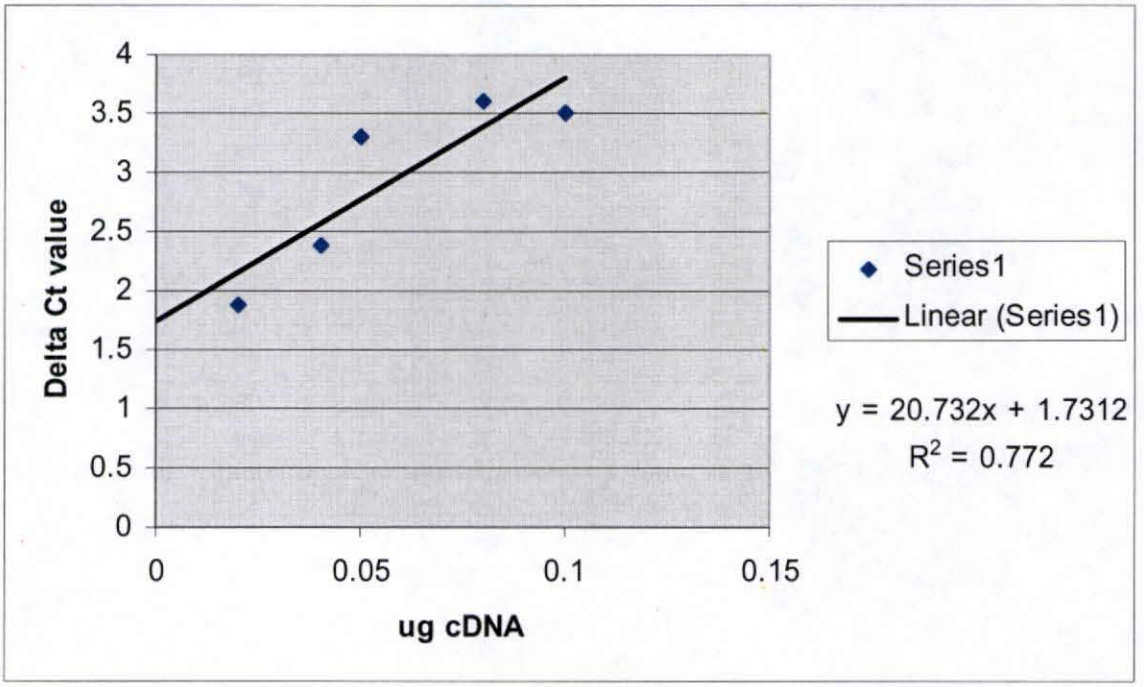
APPENDIX 2. Agarose gel electrophoresis of cDNA and PCR primers



Lane 1: 100kb Ladder
Epididymal Fat Tissue:
Lane 2: Adiponectin; 273bp
Lane 3: PPAR-α; 101bp
Lane 4: PPAR-γ; 100bp
Lane 5: GAPDH; 171bp
Liver Tissue:
Lane 6: AdipoR1; 287bp
Lane 7: AdipoR2; 230bp
Lane 8: GAPDH; 171bp

2% agarose gel electrophoresis gel stained with ethidium bromide demonstrating the specificity, appropriate product size, and purity of the real-time PCR primers and cDNA from epididymal fat tissue and liver tissue.

APPENDIX 3. Standard curve showing delta Ct value vs. µg cDNA



Depicts the standard curve which was run to determine the adequacy of 0.05 µg cDNA for the real-time PCR reaction. Samples were run in duplicate and are presented as the mean of the samples.

APPENDIX 4. Δ Ct values for Adiponectin, PPAR-α, and PPAR-γ in adipose tissues.

	SUBCUTANEOUS				EPIDIDYMAL				MESENTERIC			
	NORMAL FAT		HIGH FAT		NORMAL FAT		HIGH FAT		NORMAL FAT		HIGH FAT	
	WT	TG	WT	TG	WT	TG	WT	TG	WT	TG	WT	TG
Adiponectin	0.94±0.21 b,c,d,e	4.32±1.71 a	0.84±0.53 b,c,d,e	0.42±0.13 c,d,e	1.96±0.50 a,b,c,d	-0.54±0.11 d,e	2.93±0.22 a,b,c	-1.05±0.26 e	3.67±0.01 a,b	2.26±0.13 a,b,c,d	1.41±0.44 a,b,c,d,e	4.12±0.37 a
PPAR-α	4.43±0.69 a,b	3.48±0.94 b	4.42±0.27 a,b	5.03±1.30 a,b	6.63±0.42 a	4.48±0.58 a,b	5.30±0.39 a,b	5.22±0.10 a,b	2.38±0.08 a,b	2.81±0.15 a,b,c,d	4.76±0.67 a,b,c,d,e	2.75±0.25 a
PPAR-γ	3.18±0.63 a,b	1.73±1.81 b	4.71±0.58 a,b	2.00±0.28 a,b	2.67±0.18 a,b	2.29±0.24 b	4.31±0.49 a,b	1.84±0.15 b	2.71±0.22 a,b	6.13±1.23 a	2.93±0.45 a,b	2.93±0.0.45 a,b

Data is presented as means ± SEM. Within the same row, levels not connected by the same letter are significantly different

Δ Ct was quantified by Real-Time PCR.

	Statisctical Significance						
	FP	GEN	DIET	FP*GEN	GEN*DT	FP*DT	FP*GEN*DT
Adiponectin	***			***		*	***
PPAR-α	***						*
PPAR-γ				**	*	*	

FP = fat pad location, GEN = genotype, DT = diet.

Significance was determined at * p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001

APPENDIX 5. Δ Ct value for adiponectin receptors in gastrocnemius muscle and liver tissue.

		NORMAL FAT		HIGH FAT		SIGNIFICANCE		
		Wild-Type	Transgenic	Wild-type	Transgenic	GENOTYPE	DIET	GENOTYPE*DIET
MUSCLE	AdipoR1	6.92 b (5.39-8.45)	6.62 b (6.37-6.87)	6.68 a,b (7.43-7.93)	10.69 a (10.59-10.79)		**	
	AdipoR2	8.20 c (7.84-8.56)	8.58 c (8.43-8.73)	9.90 b (9.79-10.01)	11.12 a (10.76-11.48)	*	****	
LIVER	AdipoR1	7.50 c (7.24-7.76)	10.83 a,b (10.31-11.35)	11.20 a (10.89-11.51)	8.77 b,c (8.25-9.29)			***
	AdipoR2	4.61 c (4.58-4.64)	5.5 b (5.48-5.52)	4.78 c (4.63-4.93)	6.00 a (5.96-6.04)	****	**	

Data is presented as means (ranges). Muscle tissue sampled was gastrocnemius muscle. Within the same row, levels not connected by the same letter are significantly different. Δ Ct was quantified by Real-Time PCR. Significance was determined at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$