REGULATION OF GLUCOSE METABOLISM IN A HEPATIC AND MUSCLE CELL LINE BY ADIPONECTIN

Except where reference is made to the work of others, the work described in this thesis is my own or was done in collaboration with my advisory committee. This thesis does not include proprietary or classified information.

_____

Min Ding

Certificate of Approval:

_____	_____
Robert J. Kemppainen Professor Anatomy, Physiology and Pharmacology	Robert L. Judd, Chair Associate Professor Anatomy, Physiology and Pharmacology
_____	_____
Doug White Associate Professor Nutrition and Food Science	Marie W. Wooten Professor Biological Sciences

_____

Stephen L. McFarland
Acting Dean
Graduate School

REGULATION OF GLUCOSE METABOLISM IN A HEPATIC AND MUSCLE CELL LINE BY ADIPONECTIN

Min Ding

A Thesis

Submitted to

The Graduate Faculty of

Auburn University

In Partial Fulfillment of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama

Dec. 16, 2005

REGULATION OF GLUCOSE METABOLISM IN A HEPATIC AND MUSCLE CELL LINE BY ADIPONECTIN

Min Ding

_____
Signature of Author

_____
Date

VITA

Min Ding, daughter of Shannan Wang and Li Ding, was born on August 22, 1978, in Haian, Jiangsu, People's Republic of China. She graduated from Jiangsu Haian High School in 1995. She studied in Nanjing Medical University for five years and graduated with a Bachelor of Medicine degree in Clinical Medicine in July, 2000. She attended Cellular and Molecular Biosciences Graduate Program of Auburn University in August, 2002.

THESIS ABSTRACT

REGULATION OF GLUCOSE METABOLISM IN A HEPATIC AND MUSCLE CELL LINE BY ADIPONECTIN

Min Ding

Master of Science, Dec.16, 2005
(B.M., Nanjing Medical University, 2000)

104 Typed Pages

Directed by Dr. Robert L. Judd

Adiponectin is a 30-kD protein secreted by adipose tissue under normal conditions. Serum adiponectin concentrations are decreased in obesity and type 2 diabetes. AdipoR1 and AdipoR2 are two receptors of adiponectin with AdipoR1 abundantly expressed in muscle and AdipoR2 predominantly expressed in liver. Adiponectin has been demonstrated to decrease hepatic glucose production by inhibiting hepatic G-6-Pase and PEPCK mRNA expression, but its effects on hepatic glycogen synthesis have not been examined. In addition, eukaryotically and bacterially expressed adiponectin may have different effects on glucose metabolism due to some important structural differences. In this study, we investigated the effects of eukaryotically and

bacterially expressed adiponectin on hepatic glycogen synthesis as well as the mechanisms involved in this process. We also compared the effects of these two sources of adiponectin on muscle basal glucose uptake. A human hepatoma cell line (HepG2) and a rat ventricular cell line (H9c2) were utilized in our experiments. Hepatic basal and insulin-stimulated glycogen synthesis was evaluated in HepG2 cells treated for 3 h with eukaryotically expressed adiponectin (1 μg/ml and 5μg/ml), bacterially expressed adiponectin (20 μg/ml and 30 μg/ml), 1nM insulin and 1 nM insulin plus eukaryotically expressed adiponectin (1 μg/ml and 5μg/ml). Also, HepG2 cells were used to determine if adiponectin impacted insulin receptor signaling. Muscle glucose uptake was evaluated in H9c2 cells treated for 10 min with the same concentrations of eukaryotically and bacterially expressed adiponectin. The results indicated that AdipoR2 is present in HepG2 cells by western blotting and real-time PCR, and its expression is inhibited by insulin. AdipoR1 mRNA is also verified to be present in H9c2 cells by real-time PCR. For the metabolic effects, eukaryotically but not bacterially expressed adiponectin inhibits basal and insulin-stimulated glycogen synthesis in HepG2 cells, while bacterially but not eukaryotically expressed adiponectin stimulates basal glucose uptake in H9c2 cells. Importantly, adiponectin does not utilize the insulin signal pathway to produce the effect on hepatic glycogen synthesis, as IRS-1 was not phosphorylated by adiponectin in HepG2 cells. Our data suggest that multimer formation of higher order structure is very important for adiponectin's function in liver but not in muscle. A major effect of adiponectin on hepatic glucose metabolism may be to inhibit glycogen synthesis thereby providing more glucose for glycolysis and ATP generation.

ACKNOWLEDGMENTS

The author expresses her sincere thanks and appreciation to her major professor, Dr. Robert L. Judd for his continuous guidance, patience, understanding and great support during the entire course of this study. She also thanks Dr. Robert J. Kemppainen for providing some adiponectin, and helpful suggestions and constant support on the whole project. She expresses her sincere thanks to Ms. Qiao Zhong and Ms. Kathy O'Donnell for some experimental technical help. In addition, she thanks the other graduate committee members, Dr. Doug White and Dr. Marie M. Wooten for their guidance and support.

The author would like to thank her parents, and her brother, Xin Wang for their encouragement and support during these years. She also wishes to thank Haibo Fan for his help, encouragement and endless support. She expresses special thanks to Boshell Diabetes and Metabolic Diseases Research Program, and Auburn University Cellular and Molecular Biosciences Program for giving her the opportunity to complete the degree.

Style manual or journal used Diabetes

Computer Software used Windows XP, Microsoft Word 2000 and Excel 2000

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Overview of glucose metabolism

Glucose is a very important molecule in metabolism, both as a fuel and as a precursor of essential structural carbohydrates and other biomolecules. There are two pathways involved in glucose metabolism: anabolism (synthesis) and catabolism (degradation) pathways (Figure 1) (1). Gluconeogenesis and glycogenesis are anabolic pathways, during which glucose and glycogen are synthesized. Glycolysis and glycogenolysis belong to catabolic pathways, during which pyruvate and glucose-6-phosphate are generated. Under aerobic conditions, pyruvate can be oxidized to acetyl-CoA, which enters citric acid cycle. This cycle marks the "hub" of the metabolic system: It accounts for the major portion of carbohydrate, fatty acid, and amino acid oxidation and generates a lot of biosynthetic precursors.

Endogenous glucose production is the sum of gluconeogenesis and glycogenolysis, both feed the glucose-6-phosphate pool, which is the immediate precursor of the glucose molecules produced by glucose-6-phosphatase and released by liver and kidney into the blood stream. Glutamine is the preferential substrate for renal glucose production accounted for primarily by gluconeogenesis (2). Hepatic

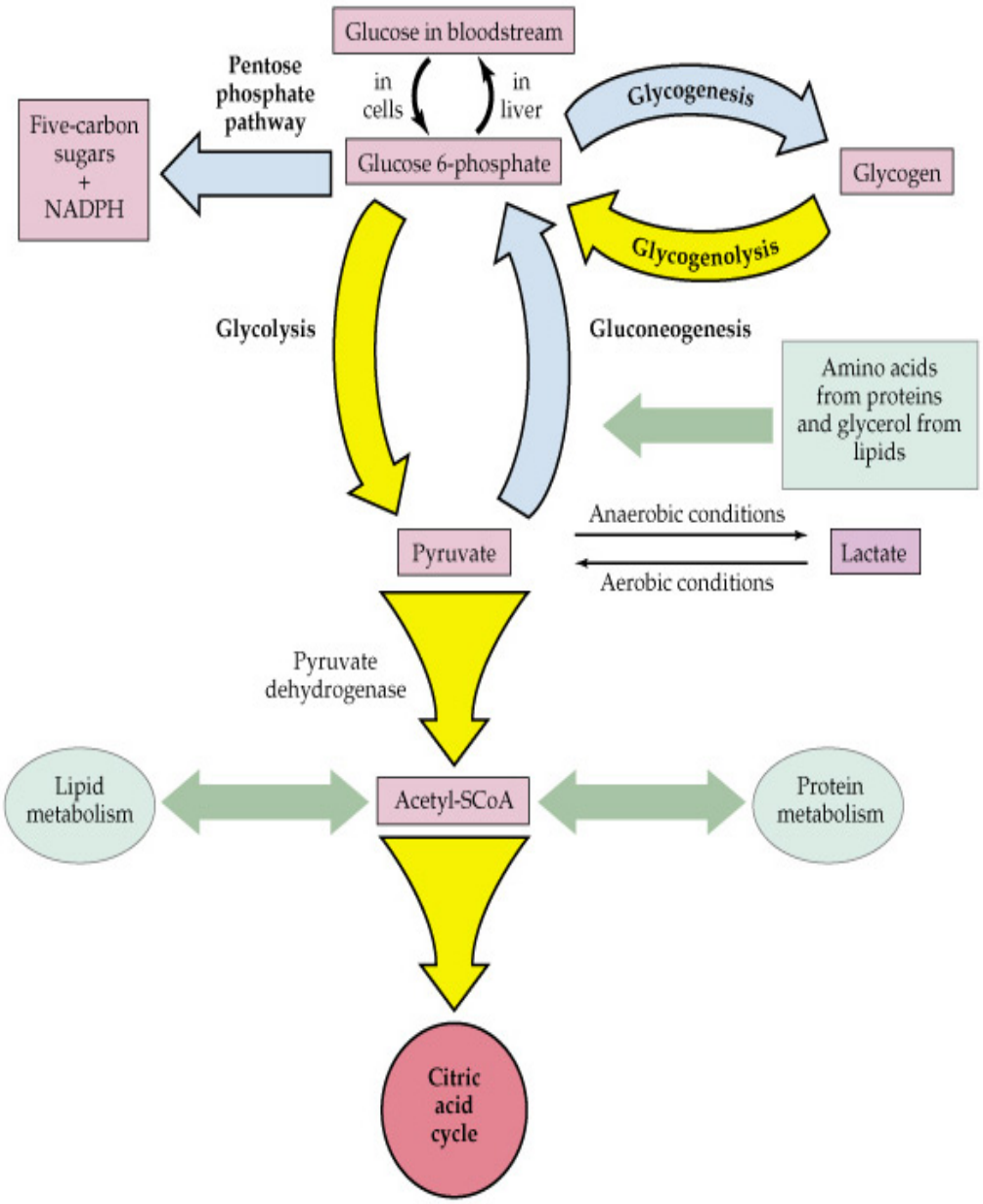


Figure 1: Overview of glucose metabolism (McMurry and Castellion, *Fundamentals of General, Organic, and Biological Chemistry*, 3rd Edition).

gluconeogenesis utilizes amino acids from proteins and glycerol from lipids, as well as lactate and pyruvate from muscle release as substrates. The cori cycle describes a connection between muscle metabolism of gluconeogenic precursors and liver gluconeogenesis. In this cycle, lactate is produced by glycolysis in muscle and transported to the liver for glucose production. The bloodstream then carries the glucose back to the muscles, where it may be stored as glycogen and used on demand by glycogenolysis and glycolysis. Hepatic gluconeogenesis is regulated by a number of enzymes. For example, the conversion of pyruvate into glucose is catalyzed by pyruvate carboxylase - phosphoenolpyruvate carboxylase (PEPCK), fructose bisphosphatase (FBPase) and glucose-6-phosphatase (G-6-Pase). These enzymes are also significantly involved in the effects of some hormones on hepatic gluconeogenesis. A widespread view holds that hepatic gluconeogenesis is primarily responsible for fasting and post-meal hyperglycemia in type 2 diabetic patients (3).

Another important anabolic pathway is glycogenesis. Muscles contain 1-2% and liver cells consist of 10% glycogen by weight, which serves as huge energy storage for the whole body. The enzymes involved in glycogen synthesis are UDP-glucose pyrophosphorylase, glycogen synthase and glycogen branching enzyme, some of which is confirmed to be genetically deficient in disease states. It should also be noted that insulin-stimulated glycogenesis has been observed to be impaired in insulin-resistant type 2 diabetes (3).

Insulin plays an important role in the regulation of hepatic glucose metabolism. It stimulates the utilization and storage of glucose as lipid and glycogen and reduces glucose synthesis and release, which is accomplished through a coordinated regulation of enzyme synthesis and activity. Insulin can also stimulate the expression of genes encoding glycolytic and fatty-acid synthetic enzymes, such as glucokinase, phosphofructokinase and pyruvate kinase, while inhibiting the expression of those encoding gluconeogenic enzymes. These effects are mediated by a series of transcription factors and co-factors, including sterol regulatory element-binding protein (SREBP)-1, hepatic nuclear factor (HNF)-4, the forkhead protein family (Fox) and PPAR co-activator 1 (PGC1). In addition, insulin regulates the activities of some other enzymes, including glycogen synthase and citrate lyase, through alterations in their phosphorylation state. All these actions lead to a direct hypoglycemic effect of insulin.

Recently research revealed that besides insulin, there are many other proteins that also affect the expression of enzymes and transcription factors involved in glucose metabolism. Adiponectin, a cytokine secreted exclusively from adipose tissue (adipokine), is one of such proteins. The purpose of this study is to investigate the role of adiponectin in the regulation of glucose metabolism.

Alterations of glucose metabolism in type 2 diabetes

Type 2 diabetes mellitus is a group of disorders characterized by hyperglycemia and associated with microvascular (optic, renal, and possibly neuropathic) and macrovascular (coronary and peripheral vascular) complications. It is also characterized by insulin resistance, possibly due to a beta-cell defect and/or changes in tissue insulin responsiveness. The primary alterations of glucose metabolism in type 2 diabetes are first in insulin-mediated suppression of endogenous glucose production and insulin-dependent stimulation of glucose utilization. Gluconeogenesis, glucose transport, glucose phosphorylation, glycogenesis and glucose oxidation are insulin-resistant. Also the insulin-independent routes of glucose metabolism may be changed. In addition, abnormal lipid metabolism is likely to play a prominent role to cause hyperglycemia in type 2 diabetes (3).

Since the insulin-dependent and independent glucose metabolism process may be significantly changed in type 2 diabetes, further research needs to be performed to identify new drugs or agents that can improve hyperglycemia and insulin resistance. Recently one study confirmed that replenishment of adiponectin, an adipocyte secretory protein, represents a novel treatment strategy for insulin resistance and type 2 diabetes (4).

Introduction of adipocyte secretory products and adiponectin

Adipose tissue is composed mainly of lipid-filled fat cells surrounded by immune cells, fibroblasts, blood vessels and collagen fibers (5). These lipid-filled fat cells are named adipocytes. Based on histology, adipose tissue can be divided into two types, white and brown adipose tissue, with white adipose tissue the predominant type in humans (6).

White adipose tissue is now identified not only to be an energy storage organ but also an active hormone system involved in the regulation of metabolism. It secretes many products named "adipocytokines" ("adipokines"), including leptin, TNF-α (tumor necrosis factor-α), IL-6 (interleukin-6), PAI-1 (plasminogen activator inhibitor-1), adipsin, adiponectin, resistin, and visfatin - the functions and effects of which are listed in Table 1 (5;7;8). These adipokines play an important autocrine role in white adipose tissue physiology and participate in the energy and glucose metabolism of the whole body.

Since adipocyte secretory products can influence both local adipocyte physiology and distant organ system metabolism, such as the central nervous system, liver, pancreas and skeletal muscle, adipose tissue disorders may sometimes cause insulin resistance and its complications, including obesity, diabetes mellitus, hypertriglyceridemia, low levels of high-density lipoprotein cholesterol, as well as hepatic steatosis (9). Studies of adipose tissue biology are very important as cases of obesity and diabetes are increasing in number all over the world. Among all those adipokines, adiponectin has recently been

Table 1: Functions and effects of adipocyte secretory proteins (Burrell *et al.*, *Am J Physiol Endocrinol Metab.*, 280:E827-E847, 2001, modified)

Molecule	Functions/Effects
Leptin	Signals to the brain regarding body fat stores Regulates appetite and energy expenditure
TNF-α	Interferes with insulin receptor signaling and may cause the development of insulin resistance in obesity
IL-6	Implicated in host defense and in glucose and lipid metabolism
PAI-1	Promotes thrombosis by inhibiting endogenous fibrinolytic system
Tissue factor	Major cellular initiator of the coagulation cascade
Angiotensinogen	Precursor of angiotensin II Regulator of blood pressure and electrolyte homeostasis
Adipsin	Possible link between the activation of the alternative complement pathway and adipose tissue metabolism
ASP	Influences the rate of triacylglycerol synthesis in adipose tissue
Adipophilin	May be a specific marker for lipid accumulation in the cells
Adiponectin	**Regulates intermediary metabolism and may be involved in regulating insulin-resistance, inflammation, angiogenesis and atherosclerosis**
Resistin	May play a role in insulin resistance
PGI_2 and $GF_{2\alpha}$	Implicated in regulatory functions such as inflammation and blood clotting, ovulation, menstruation, and acid secretion
TGFβ	Regulates a wide variety of biological responses, including proliferation, differentiation, apoptosis, and development
IGF-1	Stimulates proliferation of a wide variety of cells and mediates many of the effects of growth hormone
MIF	Involved in proinflammatory processes and immunoregulation
Visfatin	Mimics the effects of insulin and activates the insulin receptor

identified as a novel insulin-sensitizing protein with anti-inflammatory, anti-angiogenic, anti-atherosclerotic, and anti-tumor properties (10). Unlike many other adipokines, serum adiponectin concentrations are decreased in obesity and type 2 diabetes. Also, hypoadiponectinaemia has been associated with lipodystrophy, insulin resistance, and cardiovascular diseases. Therefore, this protein may provide a critical link between obesity, type 2 diabetes, and insulin resistance. In addition, several studies have demonstrated that adiponectin participates in the regulation of whole-body glucose homeostasis by decreasing hepatic glucose output. Adiponectin concentrations also inversely correlate with basal and insulin-suppressed endogenous glucose production in humans (11). These findings further support the possibility that adiponectin may be a novel treatment strategy for insulin resistance and type 2 diabetes.

Objectives of this study

Obesity is a significant public health crisis both in the United States and in the other developed countries. A recent study conducted by the Centers for Disease Control and Prevention confirms that 56.4% of Americans are either overweight or obese, and the incidence of obesity has increased 61% since 1991. Obesity is considered to be a major risk factor for the development of insulin-resistance and type 2 diabetes. More than 18 million Americans (6.3% of the population) have type 2 diabetes and about 5 million don't know that they have this disease. Another 41 million have "prediabetes" (impared

glucose tolerance). The problems behind these numbers are even more alarming. Diabetes is the leading cause of blindness and kidney failure among adults. It also causes mild to severe nerve damage coupled with circulatory problems, often leading to lower extremity amputates. Moreover, it significantly increases the risk of heart diseases. Based on statistical data, diabetes is found to be the sixth leading cause of death in the U.S., directly causing almost 60,000 deaths every year and contributing to thousands more.

The facts listed above show that obesity and type 2 diabetes mellitus are becoming more and more prevalent in the U.S. and all over the world. However, the pathophysiological link among obesity, insulin resistance and diabetes has not been clearly identified, which is critical to providing better treatment and possibly a cure for all of these disorders.

Adipose tissue is now considered an organ capable of producing a number of hormones affecting energy intake and expenditure, as well as carbohydrate and lipid metabolism, including nutrient partitioning and fuel selection. Adiponectin has insulin-sensitizing properties and may provide a link between obesity, insulin-resistance and type 2 diabetes mellitus. As mentioned, this protein can affect the expression of enzymes involved in hepatic glucose metabolism. It has been found to inhibit the expression of hepatic gluconeogenic enzymes (PEPCK, G-6-Pase) and the rate of endogenous glucose production, but its effects on hepatic glycogen synthesis have not been investigated yet. Since type 2 diabetic patients have significant glucose metabolism alterations compared

with normal persons, the present study focuses on the regulation of glucose metabolism in liver and muscle by adiponectin.

The primary objective of this study is to investigate the effects of adiponectin on hepatic basal and insulin-stimulated glycogen synthesis, as well as the mechanisms involved in this process. Recombinant mouse adiponectin expressed in HEK293-T cells and in *E.Coli* were used in this study. Since they might have different effects on glucose metabolism due to some important structural differences, another important aim of this study is to compare the effects of eukaryotically and bacterially expressed adiponectin on hepatic basal glycogen synthesis and muscle basal glucose uptake. In addition, adiponectin receptor studies are included investigating the effect of insulin on adiponectin receptor expression.

LITERATURE REVIEW

Molecular mechanisms of obesity and insulin resistance

Obesity, type 2 diabetes and insulin resistance are closely related. Obesity is defined as increased mass of adipose tissue, which confers a high risk of metabolic disorders such as diabetes and hyperlipidemia, as well as cardiovascular diseases (12). A common characteristic of both obesity and type 2 diabetes is impaired glucose and lipid metabolism, which leads to increased lipid storage in some insulin-targeted organs such as liver and muscle, thus leading to insulin resistance. Recently, research focused on the function of adipocyte has been performed to investigate the molecular mechanisms of obesity and insulin-resistance. Studies confirmed that pharmacological inhibitors of the transcription factor PPARγ / RXR (peroxisome proliferator-activated receptorγ / retinoid X receptor) can improve high-fat diet-induced insulin resistance in type 2 diabetes (4), suggesting the possible role of nuclear receptor in the development of insulin resistance. CBP protein (CREB binding protein), a transcriptional coactivator with intrinsic histone acetyltransferase (HAT) activity, may also be involved in the molecular mechanisms of obesity and insulin resistance. Studies have found that heterozygous CBP protein deficiency decreases the production of molecules causing insulin resistance and increases

the insulin-sensitizing hormones such as leptin and adiponectin, therefore preventing adipocyte hypertrophy and increasing insulin sensitivity (12). In addition, SNPs (single nucleotide polymorphisms) may lead to the development of insulin resistance and type 2 diabetes. A SNP is a single base substitution of one nucleotide with another, where both versions are observed in the general population at a frequency greater than 1%. Subjects with the G/G genotype of SNP276 in the adiponectin gene were found at increased risk for type 2 diabetes compared with those having the T/T genotype (4).

In summary, the above studies suggest that nuclear receptor and transcription coactivators may affect adipocyte function, resulting in the production of a series of adipokines which eventually leads to the development of insulin resistance and type 2 diabetes. Among those adipokines, adiponectin is a very important insulin-sensitizing protein, and a SNP in the adiponectin gene is also significantly involved in developing obesity and type 2 diabetes.

Discovery of adiponectin

Adiponectin is a 30-kDa protein secreted predominantly by adipose tissue under normal conditions. It is very abundant in plasma, accounting for 0.01% of total plasma proteins in humans (13) and 0.05% in rodents (14). Plasma concentrations of adiponectin range from 3 to 30 μg/mL across species (15). Adiponectin was discovered by four different groups using different experimental methods, and therefore it has other four

different names: apM1, Acrp30, adipoQ and GBP28. In 1995, Scherer *et al.* cloned mouse adiponectin cDNA from 3T3-L1 adipocytes for the first time and found that adiponectin mRNA was significantly upregulated during differentiation of preadipocytes to adipocytes. The respective protein was named Acrp30 (adipocyte complement-related protein of 30 kDa) (16). In the following year, a Japanese group isolated adiponectin cDNA from human adipose tissue library and named the corresponding protein apM1 (Adipose Most Abundant gene transcript) (17). Another group then used mRNA differential display to identify adiponectin gene in rats and mice and termed it adipoQ (14). Human adiponectin protein was isolated and purified from human plasma by using its affinity to gelatin-Cellulofine and named GBP28 (gelatin binding protein of 28 kDa) (18). In 1999, a Japanese group suggested a common name for this protein-adiponectin (19). Adiponectin is the currently most popular nomenclature used for this protein and is how this protein will be identified throughout this text.

Structure and function studies of adiponectin

Adiponectin is encoded by gene APM1 located on the chromosome 3q27 locus (20), which has recently been identified as a novel susceptible locus for early-onset diabetes and the metabolic syndrome (21). Human adiponectin contains 244 amino acid residues (17), while mouse adiponectin consists of 247 amino acids (16). This protein has four domains: a N-terminal signal sequence, a non-homologous domain, a collagen-like

domain and a C-terminal globular domain (Figure 2) (14;17). It shares sequence homology with type VIII and type X collagens, complement factor C1q, precerebellin and the rodent hibernation-regulated proteins hib 20, 25, and 27 (22). The three-dimensional structure of adiponectin's C-terminal globular domain is very similar to the trimeric β-stranded jellyroll topology of TNF-α (23).

Structurally adiponectin has been found to form characteristic multimers, including monomers, dimers, trimers, hexamer forms and multimer forms larger than hexamers. Scherer *et al.* demonstrated that there are two discrete complexes of adiponectin in the serum: a hexamer of about 180 kDa (LMW [low molecular weight] form) and a higher order complex of about 400 kDa (HMW [high molecular weight] form) (24). These two complexes are very stable both *in vitro* and *in vivo* and they do not spontaneously interchange in the serum. Some gender differences in the multimer formation of adiponectin have also been demonstrated. For example, females tend to have a higher total circulating adiponectin level than males. Also male mice have the majority of their adiponectin circulating as hexamers, whereas female mice have a more balanced distribution of the LMW and HMW forms (24).

Since adiponectin forms different multimers in the serum, what is responsible for the formation of multimers? Structurally the oligomer formation of adiponectin might depend on disulfide bond formation mediated by the N-terminal Cys-39 (24;25). Lodish *et al.* suggested that interchain disulfide bonds formed by residue Cys-22 may also be

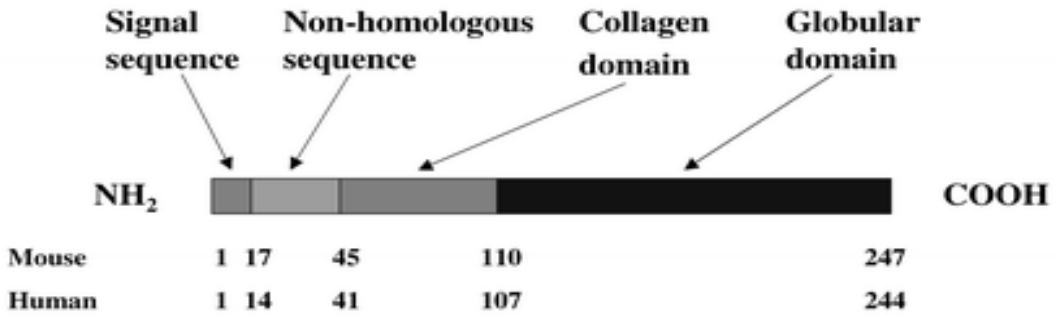


Figure 2: Structure of mouse and human adiponectin (Scherer *et al.*, *J Biol Chem.*, 270(45):26746-9, 1995, modified).

necessary for the oligomerization of adiponectin beyond the basic trimer, as well as the formation of hexamer and HMW multimers seen in freeze etch electron microscopy (26). As a whole picture of adiponectin multimer complex, it was speculated that the carboxyl terminus of adiponectin might be outside and the amino terminus inside, which was connected by the amino-terminal disulfide bond (25).

Studies also demonstrated that different adiponectin multimer species can activate different signal transduction pathways in different organs. Only trimeric, hexameric and HMW forms of adiponectin activate AMPK (AMP-activated protein kinase) pathway in hepatocytes; while in myocytes, globular domain of adiponectin can activate AMPK pathway. AMPK is the downstream component of a protein kinase cascade that acts as an intracellular energy sensor maintaining the energy balance within a cell. The increased AMPK by adiponectin leads to inhibition of hepatic glucose production, reduction of intracellular triglycerides, amelioration of hepatic insulin resistance and enhancement of muscle glucose uptake (Figure 3) (25).

Lodish *et al.* studied adiponectin and the regulated signal transduction pathways (26). They suggested that trimeric adiponectin could activate AMPK, and hexameric and HMW forms of adiponectin activated NF-κB (nuclear factor kappa B) (26). The biological significance of NF-κB activation is still not very clear. In addition, a recent study done by Matsuzawa *et al.* revealed that only HMW adiponectin selectively suppressed endothelial cell apoptosis, whereas the LMW form did not have this effect

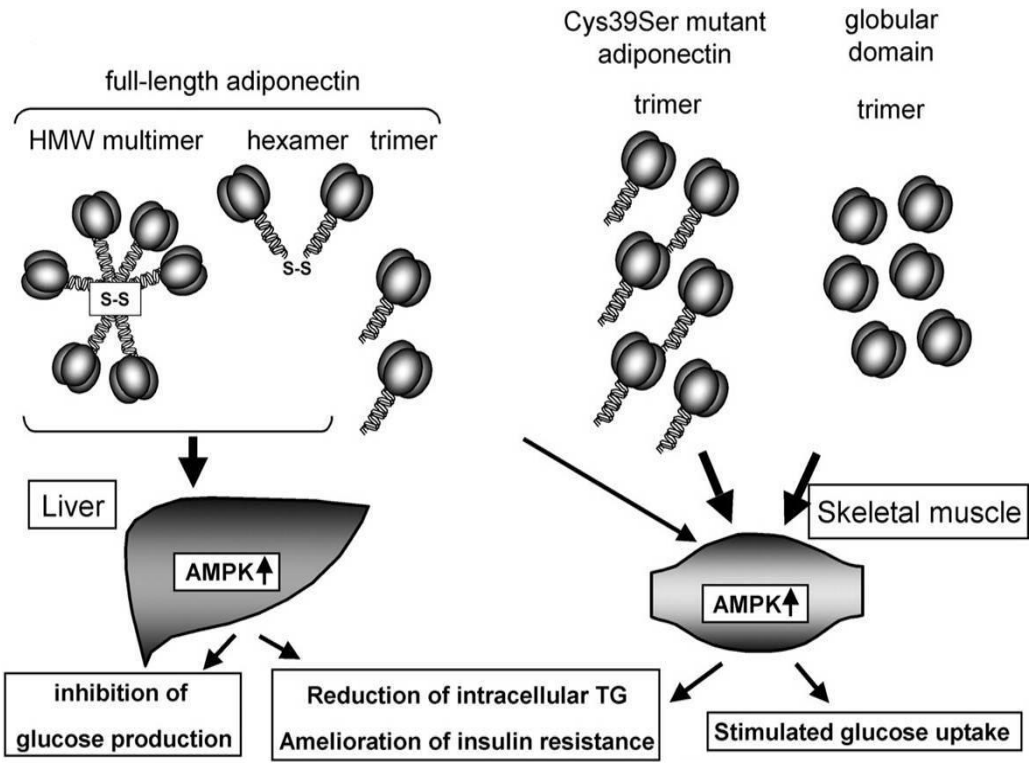


Figure 3: Different forms of adiponectin activate AMPK pathway in liver and skeletal muscle (Kadowaki *et al.*, *J Biol Chem.*, 278:40352-40363, 2003).

(27). All these studies appear to demonstrate that HMW form of adiponectin is the most biologically active form.

As for the relationship of its structure and function, the hydroxylation and glycosylation of the four conserved lysine residues in the collagen domain of adiponectin are also very important. These modifications have been reported to be involved in the inhibition effect of adiponectin on hepatic glucose production. Hydroxylation and glycosylation-deficient adiponectin produced in *E.coli* exhibits a decreased ability in inhibiting hepatic glucose production (28).

Based on the findings that different forms of adiponectin have different biological activity, Scherer *et al.* recently proposed a new index that takes the different circulating forms of serum adiponectin into account and called this index the adiponectin sensitivity index (S_A). It is defined as the percentage of the HMW forms compared to the total circulating adiponectin (29). This index may help to assess a patient's hepatic insulin sensitivity in combination with other parameters, such as oral glucose tolerance tests and and the measurement of inflammatory cytokines, etc.

Adiponectin gene expression and regulation

Adiponectin is a very abundant protein in plasma. It is secreted exclusively by adipose tissue under normal conditions (16). However, adiponectin mRNA was also detectable in mouse hepatocytes after the treatment with carbon tetrachloride, and in

human hepatoma cell line (HepG2) when treated with IL-6 (30). In addition, transcription, translation and secretion of adiponectin have been confirmed in human osteoblasts (31). In that study, adiponectin was found to play a functional role in bone homeostasis, which suggested that this protein might be an important signal linking fat and body weight to bone density (31).

There are several mechanisms that regulate the synthesis and secretion of adiponectin. Insulin stimulates, while IL-6, Forskolin, TNF, and dexamethasone inhibit adiponectin gene expression and secretion from 3T3-L1 adipocytes (16;32). Peroxisome proliferators-activated receptors (PPAR), which belong to the nuclear hormone receptor superfamily, also play a role in the regulation of adiponectin synthesis. PPAR α agonists (ie. fibrates) decrease adiponectin mRNA in white adipose tissue of obese db/db mice which lacks functional leptin receptors (33), whereas PPAR γ agonists (ie. thiazolidinediones) stimulate adiponectin gene expression in obese mice and insulin-resistant obese humans (34). In addition, stimulation of the cAMP-PKA pathway by the β-adrenergic agonist isoproterenol or the synthetic cAMP analogue dibutyryl-cAMP reduces the level of adiponectin mRNA in 3T3-L1 adipocytes (35;36). Since adipose tissue is very sensitive to oxidative stress, this can also decrease adiponectin secretion in 3T3-L1 adipocytes (37). Another very important regulator is testosterone. Research has shown that testosterone decreases adiponectin secretion in 3T3-L1 adipocytes, and reduces plasma adiponectin concentration in both sham-operated

and castrated male mice (38). Importantly, this hormone selectively inhibits high molecular weight form of adiponectin secreted from rat adipocytes (39). This might be able to explain why men tend to have lower circulating levels of adiponectin than women, and HMW multimers are significantly more abundant in female subjects than in male subjects.

In some disease conditions, serum adiponectin concentrations often change compared with the normal situation. Studies have demonstrated increased serum adiponectin level in anorexia nervosa, type 1diabetes and chronic renal failure. However, in obesity, type 2 diabetes, the metabolic syndrome and coronary artery disease, serum adiponectin level is decreased (40). The pathophysiological mechanisms responsible for these changes are still not very clear.

Adiponectin receptors

Adiponectin has a number of important biological functions, including changing hepatic gene expression, modulating the endothelial cell inflammatory response, regulating immune cell function and suppressing macrophage-to-foam cell transformation, etc. In 2003, Kadowaki *et al.* cloned two adiponectin receptors in mouse, which were named AdipoR1 and AdipoR2. AdipoR1 is abundantly expressed in skeletal muscle as a high-affinity receptor for globular adiponectin and a low-affinity receptor for full-length adiponectin; AdipoR2 is expressed predominantly in liver as an intermediate-affinity

receptor for both forms of adiponectin (41). These two receptors are distantly related to the family of seven-transmembrane spanning G-protein-coupled receptors. Since they have an inverted topology with the N-terminus intracellular portion, and their extracellular domain is small, they are distinct from the seven-transmembrane spanning receptors (42). These two receptors can mediate increased AMPK and PPAR-α ligand activities, as well as fatty-acid oxidation and glucose uptake in the presence of adiponectin (41).

Besides AdipoR1 and AdipoR2, T-cadherin was recently identified to be another receptor for hexameric and HMW forms of adiponectin (42). Cadherins are a superfamily of adhesion molecules that mediate Ca^{2+}dependent cell–cell adhesion in all solid tissues of the organism (43). T-cadherin is an atypical member of the cadherin family which lacks both the transmembrane and cytoplasmic domains. The only known function of T-cadherin is to participate in the regulation of neuron growth during embryogenesis (44), and it is expressed only in endothelial and smooth muscle cells, but not in hepatocytes. Since liver is an important target for adiponectin, maybe some other cell types in the liver such as fibroblast, endotheial or blood derived cells have this receptor so that hexameric and high molecular weight forms of adiponectin can work in the liver (42). The detailed function and signal downstream of this receptor are not very clear yet.

AdipoR1/R2 receptor gene expression and regulation

In addition to the two target organs, liver and muscle, AdipoR1 and AdipoR2 receptors are also expressed in other cell types. For instance, expression of both receptors was reported at high levels in human and rat pancreatic β cells, and their expression regulation was suggested to be a mechanism modulating the effects of circulating adiponectin (45). Also, bone-forming cells express both AdipoR1 and AdipoR2 (31). Moreover, it was reported that these two receptors are expressed in brown and white adipocytes of lean mice, which mediate paracrine effects of adiponectin in adipose tissue (46).

Like adiponectin itself, AdipoR1 and AdipoR2 gene expression has been found to be regulated under different conditions both *in vitro* and *in vivo*. AdipoR2 synthesis is significantly increased during the differentiation of 3T3-L1 preadipocytes, and its mRNA expression can also be induced by growth hormone in 3T3-L1 adipocytes (47). On the other hand, TNF-α downregulates AdipoR2, but not AdipoR1 mRNA levels in pig stromal-vascular cell culture (48). In addition, a parallel circadian gene expression pattern for both AdipoR1 and AdipoR2 was recently identified, with lower expression at night and higher expression during the day in brown, epigonadal and subcutaneous adipose tissue (46).

Another very important agent that is able to regulate adiponectin receptor gene expression is insulin. It was reported that the expression of AdipoR1 and/or AdipoR2

appears to be inversely regulated by insulin in some physiological and pathophysiological conditions, such as fasting/refeeding, insulin deficiency and hyperinsulinemia, and may also be correlated with adiponectin sensitivity. Recently Kadowaki *et al.* demonstrated that the expression of AdipoR1/R2 in skeletal muscle and liver were significantly increased in fasted mice with lower circulating insulin levels, and decreased in refed mice with higher insulin levels. STZ-induced insulin deficiency increased and the insulin replenishment reduced the expression of AdipoR1/R2 *in vivo*. Insulin might also decrease AdipoR1/R2 gene expression via the PI3-kinase/Foxol 1 pathway *in vitro* (49). Moreover, the expression of AdipoR1/R2 in insulin-resistant ob/ob mice were significantly decreased in skeletal muscle and adipose tissue, which were correlated with decreased adiponectin binding to membrane fractions of skeletal muscle as well as decreased AMPK activation by adiponectin. These decreased adiponectin effects are termed "adiponectin resistance" (49).

On the other hand, only AdipoR1 mRNA concentrations are increased in muscles of STZ induced diabetic mice and decreased in type 2 diabetic db/db mice, while hepatic AdipoR2 expression was not significantly changed in either STZ or genetically obese mice (50). This is almost the same as the findings of several other groups, all of which suggested that regulation of AdipoR1, but not that of AdipoR2 may be involved in glucose and lipid metabolism in diabetic states (50-53).

Physiological functions and mechanisms of adiponectin

Adiponectin predominantly has three physiological functions, the most important of which is its insulin-sensitizing property. In addition, it has anti-inflammatory and anti-atherogenic functions (54). Recently it has been confirmed that this protein may also have anti-angiogenic and anti-tumor functions (55). These properties have made this novel adipocytokine a promising therapeutic tool for the future.

The insulin-sensitizing function of adiponectin has been suggested by several studies. In Pima Indians, Caucasians and Asians, hypoadiponectinemia was more closely linked to hyperinsulinaemia and insulin resistance than to adiposity (56-58). Importantly, low basal adiponectin concentrations in Pima Indians predicted the decreased insulin sensitivity independent of adiposity (58). In addition, studies performed in Rhesus monkeys clearly indicated that the drop in adiponectin levels preceded the development of hyperinsulinaemia (59;60), which suggested that low adiponectin levels might be a cause and not a consequence of hyperinsulinaemia.

There are four possible mechanisms involved in the insulin-sensitizing property of adiponectin: 1) increased lipid oxidation; 2) direct improvement of insulin signaling at the receptor/post-receptor level; 3) change of glucose metabolism in the liver; and 4) inhibition of TNF-α signaling in adipose tissue (61). First, since both circulating fatty acids and tissue triglycerides are involved in the pathogenesis of insulin resistance, increased fatty acid catabolism mediated by adiponectin improves insulin sensitivity (62).

Second, increased insulin-induced tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and of protein kinase B in skeletal muscle by adiponectin improves insulin sensitivity (63). In addition, Combs *et al.* have observed that intravenous adiponectin infusion had no effect on glucose uptake, glycolysis and glycogen synthesis, but decreased hepatic glucose production by reducing the expression of enzymes involved in gluconeogenesis (64). Furthermore, some data appear to indicate that adiponectin can reduce both TNF-α secretion and signaling in macrophages/vascular endothelial cells, this reduced activity of TNF-α could improve insulin sensitivity in adipose tissue also (65).

Another important function of adiponectin is that it has anti-inflammatory property. It might suppress the activation of transcription factor NF-κB in vascular endothelium, which is involved in inflammation. Since this activation is mediated by TNF-α and accompanied by accumulation of cAMP, and is blocked by adenyl cyclase and protein kinase A inhibitors, adiponectin might modulate the endothelial cell inflammatory response via cAMP-PKA and NF-κB pathways (66). These pathways may also play a major role in the adhesion of adiponectin to the damaged vessel wall.

Since adiponectin can suppress the expression of adhesion molecules in vascular endothelial cells and the production of cytokine from macrophages, it will inhibit the inflammatory processes that occur during the early phases of atherosclerosis. Therefore, it is possible that hypoadiponectinemia may cause the development of atherosclerotic

vascular disease (54).

Molecular action of adiponectin in important organs

As mentioned, serum adiponectin level often changes in some disease states. For instance, decreased circulating adiponectin level has been found in obesity, insulin resistance, hyperinsulinemia and type 2 diabetes, and adiponectin concentrations can increase concomitantly with weight loss. This negative correlation between adiponectin and visceral adiposity is very unique. Studies have demonstrated that the synthesis and secretion of adiponectin is mostly regulated in adipocytes. Under normal conditions, small adipocytes secrete insulin-sensitizing hormones, such as adiponectin and leptin. Adipocyte hypertrophy induced by high-fat diet decreases the production of adiponectin and increases leptin, TNF-α and resistin secretion, among which the increased production of TNF-α and resistin leads to insulin resistance in obesity. Because TNF-α expression is positively correlated with adiposity and there is evidence indicating a mutual negative regulation between TNF-α and adiponectin, TNF-α could be the adipokine responsible for the suppression of adiponectin levels when fat tissue is increased (40).

Figure 4 (67) summarizes the action of adiponectin on some important peripheral organs. Liver and skeletal muscles are the two most important target organs for adiponectin. When full-length adiponectin binds to AdipoR2 receptor in the liver, it decreases lipid synthesis and glucose production. The binding of globular adiponectin to

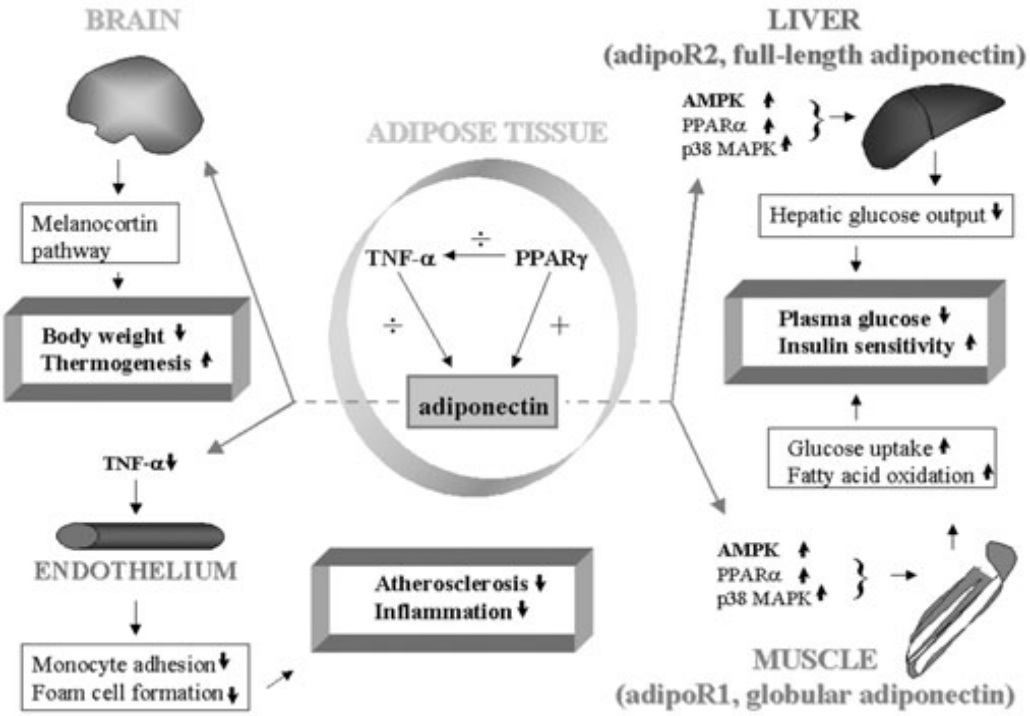


Figure 4: Molecular action of adiponectin on target tissues and other important peripheral organs (Richelsen *et al.*, *Obes.Rev.*, 6:13-21, 2005).

AdipoR1 receptor in the muscles leads to decreased triglyceride production and increased fatty acid oxidation, glucose uptake, as well as energy dissipation. These important actions in target organs produce decreased glucose and free fatty acids concentrations in the blood and increased insulin sensitivity (40). In the brain, adiponectin can activate melanocortin pathway to decrease body weight and increase thermogenesis (68). Moreover, adiponectin negatively regulates myelomonocytic progenitor cell growth and TNF-α production in macrophages, as well as suppresses macrophage-to-foam cell transformation. Also, it inhibits endothelial cell production of adhesion molecules *in vitro*, suppressing the adhesion of monocytes (69). The above two actions in macrophages and endothelial cells entitle this protein to the anti-atherosclerosis and anti-inflammation properties.

Effects of adiponectin on liver and muscle insulin sensitivity

Ever since adiponectin was discovered in 1995, studies have been performed to investigate the effects of adiponectin on hepatic glucose production. Scherer *et al.* found that full-length mammalian-cell expressed adiponectin increased insulin sensitivity in the liver, resulting in reduced hepatic glucose output and decreased serum glucose levels (70). This glucose-lowering effect of adiponectin was not associated with an increase in insulin levels. Importantly, they found that adiponectin increased the ability of sub-physiological concentrations of insulin to suppress glucose production, which suggested that this

protein might be a potent insulin enhancer connecting adipose tissue and whole-body glucose metabolism. In addition, Rossetti *et al.* did some *in vivo* studies on adiponectin. They demonstrated that adiponectin significantly inhibited glucose production and G-6-Pase flux, but the rate of glucose cycling was unchanged (64). Furthermore, infusion with recombinant adiponectin did not significantly alter the rates of hepatic glucose uptake, glycolysis and glycogen synthesis. As for the enzyme change by adiponectin, Rossetti *et al.* showed that this protein markedly decreased the levels of G-6-Pase and PEPCK mRNA expression in liver (64). This may explain in part why the rate of endogenous glucose production was inhibited by adiponectin. Besides PEPCK and G-6-Pase, Kadowaki *et al.* verified that the glucose lowering effect of adiponectin also required in part the activation of liver AMPK (71). Adiponectin activates AMPK in liver, thereby directly regulating glucose metabolism and insulin sensitivity both *in vitro* and *in vivo*.

The following graph is a model for molecular action of adiponectin in liver (Figure 5) (72). Only trimeric, hexameric and high molecular weight forms of adiponectin can increase insulin sensitivity in the liver. The binding of adiponectin to the adipoR2 receptor in hepatocytes activates AMPK and PPAR-α (peroxisome proliferator-activated receptor α), resulting in decreased expression of enzymes involved in gluconeogenesis such as PEPCK and G-6-Pase. The activation of AMPK can also lead to phosphorylation and inhibition of acetyl CoA carboxylase (ACC), an enzyme

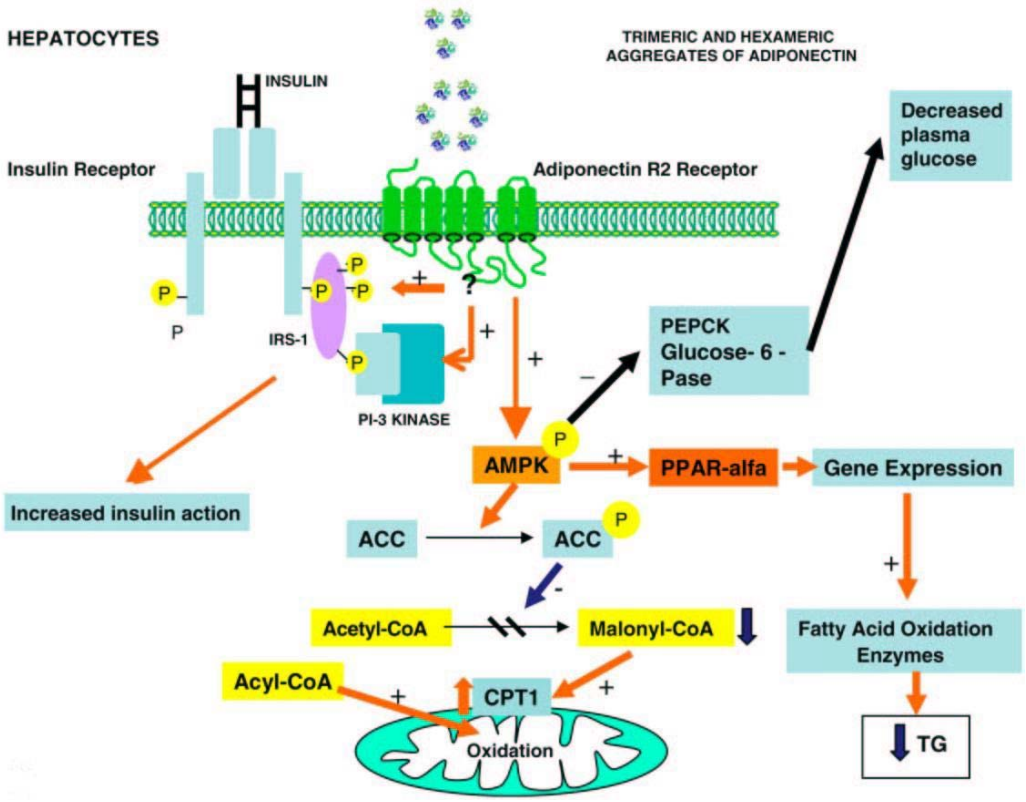


Figure 5: Model for molecular action of adiponectin in liver (Gil *et al.*, *Clin. Nutr.*, 23:963-974, 2004).

responsible for triglycerides synthesis, therefore, triglycerides synthesis is decreased and fatty acid oxidation is increased. On the other hand, full-length adiponectin may directly increase the phosphorylation of insulin receptor substrate-1 (IRS-1) and activate the phosphatidylinositol 3–kinase (PI-3 Kinase) pathway, thus increasing hepatic insulin sensitivity.

Several other studies also investigated the effects of adiponectin on muscle insulin sensitivity. Ruderman *et al.* found increased muscle fat oxidation and glucose transport by globular adiponectin. During this process, AMPK activity was also increased, and ACC was phosphorylated and inhibited (73). In addition, Sweeney *et al.* showed that globular adiponectin increased glucose uptake, GLUT4 myc translocation, and fatty acid oxidation and lactate production as well, while decreased basal and insulin-stimulated rates of glycogen synthesis in rat skeletal muscle cells (74). Also increased AMPK and decreased ACC activity by globular adiponectin were observed in these cells (74).

Figure 6 summarizes the molecular action of adiponectin in the skeletal muscle (72). Both full-length and globular adiponectin can bind to Adipo R1 receptor in muscle cells, which is associated with increased AMPK phosphorylation and p38 MAPK (mitogen-activated protein kinase) phosphorylation. The phosphorylation of AMPK causes activation of PPAR-α and inhibition of ACC so that triglycerides synthesis is decreased, fatty acid oxidation and lactate production are increased. On the other hand, phosphorylation of p38 MAPK may cause increased GLUT4 translocation and glucose

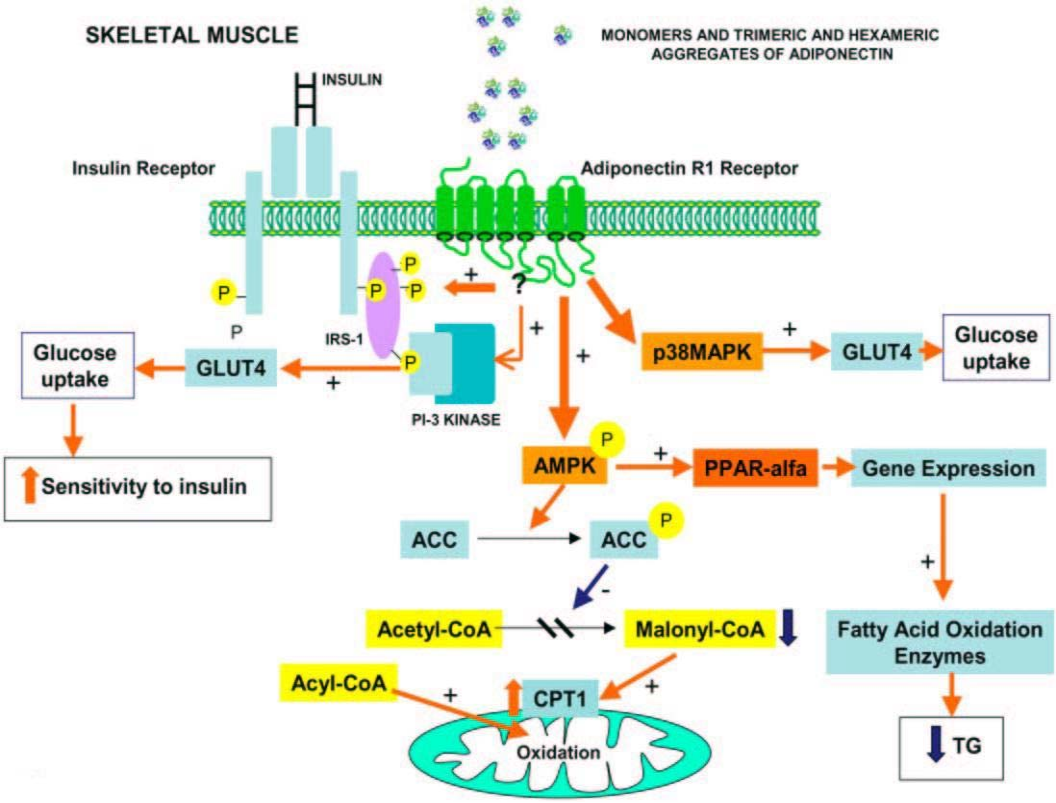


Figure 6: Model for molecular action of adiponectin in skeletal muscle (Gil *et al.*, *Clin. Nutr.*, 23:963-974, 2004).

uptake in skeletal muscle by adiponectin.

From the above literature review, we summarize that adiponectin can reduce hepatic glucose output and serum glucose levels by decreasing the expression of enzymes involved in hepatic gluconeogenesis, such as PEPCK and G-6-Pase. This glucose lowering effect may also require in part the activation of liver AMPK. In the liver, adiponectin can increase AMPK and PPAR-α ligand activities, therefore increasing hepatic insulin sensitivity. However, its effects on hepatic glycogen synthesis and energy metabolism have not been examined and will be a focus of our study. Also the mechanisms involved in this process have not yet been identified.

Structurally, adiponectin has been found to form characteristic multimers, including monomers, dimers, trimers and hexamers. These multimers have been demonstrated to activate different signal transduction pathways in different organs. Whether they also have different metabolic effects on hepatic and muscle glucose metabolism has not yet been determined and will also be a focus of our study.

MATERIALS

C57BL6J mice were obtained from Harlan (Indianapolis, IN). A human hepatoma cell line (HepG2) and a rat ventricular cell line (H9c2) were purchased from American Type Culture Collection (Manassas, VA). Recombinant eukaryotically expressed mouse adiponectin was purchased from both Apotech (San Diego, CA) and Biovendor (Candler, NC). Recombinant bacterially generated mouse adiponectin was produced by Dr. Robert J. Kemppainen. Hank's Balanced Salt Solution and streptomycin-penicillin were purchased from Mediatech Inc (Herndon, VA). Type I collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ). RPMI 1640 medium was obtained from Fisher Scientific (Pittsburgh, PA). Insulin, fetal bovine serum (FBS), gentamycin, dexamethasone, Trypan Blue, Trizol, and Dulbecco's modified Eagle's medium (DMEM) were supplied by Invitrogen (Grand Island, NY). A glucose analysis kit was purchased from Wako Chemicals (Richmond, VA). D-[^{14}C] glucose was obtained from New England Nuclear (Boston, MA). 2-[^{3}H] deoxyglucose was purchased from Perkin Elmer Life Sciences (Boston, MA). AdipoR2 antibody was purchased from Alpha Diagnostic International (San Antonio, TX). A goat anti-rabbit antibody conjugated to horseradish peroxidase was purchased from Cell Signaling (Beverley, MA). Anti-IRS-1

antibody, antiphosphotyrosine antibody (4G10) and a mouse anti-rabbit antibody conjugated to horseradish peroxidase were purchased from Upstate Biotechnology (Lake Placid, NY). Chemiluminescence reagents were obtained from Amersham Biosciences (England). All other reagents from commercial sources were of analytical grade.

METHODS

Isolation of mouse hepatocytes

C57BL6J mice (20-25g) were obtained at about 7-8 weeks old. They had access to food and water ad libitum and were maintained on a 12:12-h light/dark cycle. All experimental protocols were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) prior to initiation.

Mouse hepatocytes were isolated by modified *in situ* perfusion as described by Harman *et al*. (75). The mice were administered heparin (1000 U/kg, i.p.) 20 min before anesthetization and then anesthetized with sodium pentobarbital (250 mg/kg, i.p.). A longitudinal incision was made into the abdomen, and curved forceps were used to place and tie a ligature loosely around the vena cava just prior to the renal veins. A 22 gauge ×1 inch IV type catheter was inserted into the vena cava approximately 3-5 mm distal to the ligature. The stylet was removed and the catheter was advanced in order that its tip was anterior to the renal veins. The ligature was then gently tightened and knotted but not constricted. The infusion tubing was connected to the catheter and the infusion pump was started at low speed (approximately 3 ml/min). The portal vein was then severed and the pump flow rate was immediately increased to about 6 ml/min. The diaphragm was cut

through to expose the thoracic cavity and the anterior vena cava was clamped off between the heart and the diaphragm with a small hemostat. The liver changed from dark red-brown to a light tan color as the blood was flushed out. Then the liver was perfused in a retrograde fashion for 3 min with a modified Hank's buffer (pH =7.4, 37°C) containing 0.1mM EGTA. Perfusion was continued for a further 6 min with modified Hank's buffer containing 1mM $CaCl_2$ and 0.2 mg/ml Type I collagenase. After perfusion, the liver was excised and gently teased apart using a blunt spatula. The resulting cell suspension was filtered through two layers of nylon mesh (250 and 100 μm) to remove undigested material and placed in the 4°C refrigerator for 10 min, then washed three times with Hepatocyte Wash Medium (Invitrogen, Grand Island, NY). Cells were resuspended in RPMI 1640 medium with L-glutamine and 2000 mg/L D-glucose, 10% FBS, 50 μg/ml gentamycin, 10nM dexamethasone and 1nM insulin. Trypan blue staining was performed to count cell number and evaluate the viability of cells. Normally $5\text{-}8\times10^6$ cells were obtained from one mouse and a viability of 80% - 90% was attained. Mouse hepatocytes were then plated in 60-mm collagen I coated dishes and put in an incubator maintained at 37°C in a humidified 95% air, 5% CO_2. Cells typically adhered to the plate within 2-4 hours. Non-adherent cells were removed prior to cell culture.

Cell culture

Mouse hepatocytes were cultured in RPMI 1640 medium with L-glutamine and 2000 mg/L D-glucose, 0.4% FBS and 50 μg/ml gentamycin, and maintained at 37°C in a humidified 95% air, 5% CO_2 for one day prior to experiments. HepG2 cells were cultured in DMEM containing 5.5 mM D-glucose, 10% FBS, 100 U/ml penicillin and100 μg/ml streptomycin, and passaged every three days. For experiments, HepG2 cells were cultured in either 24-well plate or 60-mm normal dishes, grown to 70% confluence, and maintained in serum-free DMEM medium overnight. Cells were then treated with insulin and adiponectin as described. H9c2 cells were also cultured in DMEM containing 5.5 mM D-glucose, 10% FBS, 100 U/ml penicillin and100 μg/ml streptomycin. Cells were grown to about 90% confluence, culture medium was changed to complete DMEM medium containing 1% FBS and maintained for 14 days. During this process, H9c2 myoblasts differentiated into myotubes. For experiments, H9c2 myotubes were serum starved overnight in DMEM with 0.5% FBS, then treated with insulin and adiponectin as indicated. All of the above three types of cells were grown in an incubator maintained at 37°C in a humidified 95% air, 5% CO_2.

The following pictures are morphologies of primary mouse hepatocytes, HepG2 cells and H9c2 myotubes.

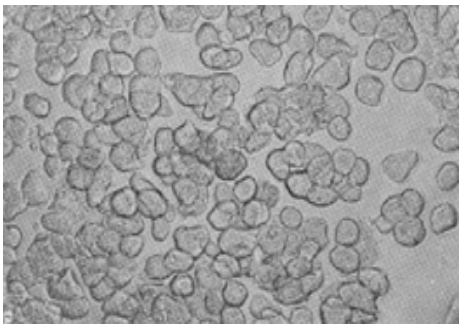
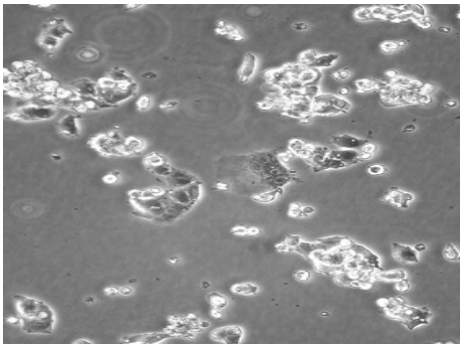
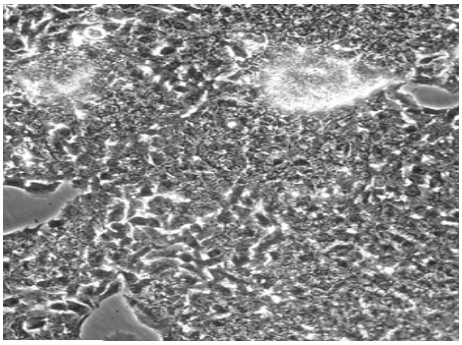


Figure 7: Primary Mouse Hepatocytes.





Low Density

High Density

Figure 8: HepG2 Cells.

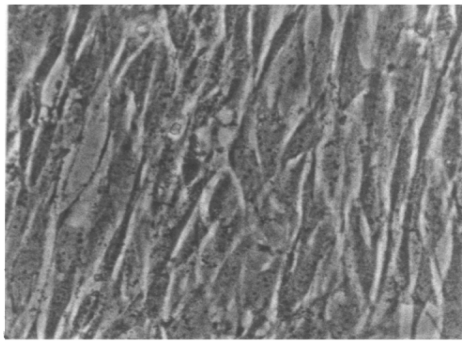


Figure 9: H9c2 Myotubes.

Production of bacterially expressed recombinant mouse adiponectin

Mouse adiponectin was cloned using reverse transcription-polymerase chain reaction (RT-PCR). Briefly, RNA was extracted from mouse epididymal fat using guanidine isothiocyanate/phenol (Trizol, Invitrogen, Grand Island, NY). Approximatey 1 ug of total RNA was used in the RT reaction. For PCR cloning, the mouse adiponectin GenBank sequence accession number (U37222) was used for primer design. Primers were designed to include amino acids 18 through 247 of the adiponectin gene and therefore did not include the signal sequence. The 693 base pair coding region was generated by PCR using primers with BamH1 and Sal1 restriction sites to facilitate cloning into plasmid pQE-80L (Qiagen, Valencia, CA). The resulting plasmid was sequenced to verify that the adioponectin sequence was correct and in frame with the 6-histidine tag encoded by pQE-80L. This plasmid was transformed into BL21-DE3 bacteria for recombinant protein production. Following overnight growth in Luria Broth (LB) containing chloramphenicol and ampicillin, the bacteria were diluted 1:50 into fresh LB and allowed to grow for an additional 3 hours. At that time, IPTG was added to a final concentration of 1 mM. Three hours later, bacteria were collected by centrifugation and their cytosolic contents extracted using B-PER reagent (Pierce Biochemical, Rockford, IL) with benzonase (Novagen, San Diego, CA). The 6-histidine-adiponectin was purified using a column of nickel-agarose with elution with increasing concentrations of imidizole. The fractions containing eluted adiponectin were pooled and dialyzed overnight in 10

mM Tris HCL, pH=7.5. The protein was mixed with glycerol (10% final vol:vol) and stored frozen in aliquots.

Measurement of glucose production

Primary mouse hepatocytes were cultured in 60-mm dishes pre-coated with type I collagen. Cells were incubated in complete RPMI 1640 medium with/without 33 pM insulin and/or 2 μg/ml of adiponectin for 24 hours. Media was removed and glucose production was measured by incubating these cells in glucose-free RPMI 1640 medium in the presence or absence of 5 mM of pyruvate for another 6 hours. The medium in each dish was then collected and a glucose analysis kit (Wako Chemicals, Richmond, VA) was utilized to analyze the glucose concentration in each group.

Isolation of HepG2 cell membranes

HepG2 cells were cultured in 60-mm dishes. Cells were rinsed with 3.0 ml of warm PBS (Mediatech Inc, Herndon, VA). After the PBS was aspirated, ice-cold Buffer A (250 mM sucrose, 20 mM HEPES, 1 mM EDTA and one tablet Complete EDTA-free protease inhibitor, pH = 7.4) was added. Cells were then scraped and transferred to a 12 ml centrifuge tube. Cell suspensions were homogenized in a glass Dounce homogenizer and supernatant was centrifuged at 30,000 g (16,000 rpm JA20 rotor) for 22 min at 4°C to yield a pellet of total cellular membranes. The cytosolic supernatant was then removed

and cell membranes were resuspended in Buffer B (20 mM HEPES, 1 mM EDTA and one tablet Complete EDTA-free protease inhibitor, pH = 7.4) for further protein assay.

Measurement of protein concentration of the HepG2 cell membrane suspensions

Bio-Rad Non-DC protein assay method (Bio-Rad, Hercules, CA) was utilized to measure protein concentration of the HepG2 cell membrane suspensions. 0.5 mg/ml, 0.4 mg/ml, 0.2 mg/ml, 0.1 mg/ml and 0.05 mg/ml of BSA (Bovine Serum Albumin) solution were prepared from the BSA stock solution (1.44 mg/ml) as standard solutions. Dye reagent was prepared by 1:4 dilution of the original concentrate. The HepG2 cell membrane samples were also diluted if necessary. 10 µl of each standard and diluted sample solution and 200 µl of the dye reagent were added into each well of a microtiter plate. After incubated at room temperature for 5 min, all of the samples and dye reagents were mixed thoroughly using a microplate mixer and the light absorbance of each sample was measured at 595 nm wavelength. Protein concentration of each cell membrane sample was calculated based on the generated standard curve.

Analysis of AdipoR2 expression by Western Blotting

Total cell membranes were prepared from HepG2 cells analyzed by western blotting for the presence of AdipoR2. In addition, cells were treated with/without insulin (1, 10, 100 nM) for 24 hours and the isolated cell membranes were analyzed by western

blotting. The processes of western blotting were as follows: 40 μg of cell membrane sample protein was separated by 10% SDS-polyacrylamide gel electrophoreses (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes in a transfer buffer containing 20 mM Tris-HCl, 154 mM glycine and 20% methanol. The membranes were blocked with 7% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T buffer, pH=7.6) and incubated overnight with a primary AdipoR2 antibody (1:500 dilution), followed by incubation with a goat anti-rabbit secondary antibody (1:1000 dilution) for one hour. Blots were developed by ECL chemiluminescence reagents. Immunoreactive bands were then visualized and the optical pixel density of the bands was determined using the Bio-Rad Quantity One Flour-S Multi-Imager System.

Isolation of total RNA from both HepG2 cells and H9c2 myotubes

HepG2 cells and H9c2 myotubes were cultured in 60-mm dishes. Cells were washed with cold PBS and 1.0 ml of Trizol was added into each dish. After the Trizol was transferred to sterile Eppendorf tubes, chloroform:isoamyl alcohol (Fluka Biochemika, Switzerland) was added and mixed by inversion. The mixture of solution was centrifuged cold at 12000 g for 15 min, top aqueous layer was removed to another clean Eppendorf tube and ice-cold 100% isopropyl alcohol was added. This mixture was again centrifuged cold at 12000 g for 10 min. Supernatant was poured off and discarded, the remaining pellet was visualized and ice-cold 75% ethanol was added to wash it. The resuspended

pellet was then centrifuged cold at 7500 g for 5 min and the acquired RNA was dissolved in RNase free water for concentration determination.

Analysis of AdipoR1/R2 expression by Real-Time PCR

Total RNA (1 μg) from either HepG2 cells or H9c2 myotubes was reverse transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-Time polymerase chain reaction (PCR) analyses were performed in the iCycler IQ Real-Time PCR detection system (Bio-Rad, Hercules, CA). The 30 μl amplification mixture contained 2 μl of cDNA, 200 nM of AdipoR1/R2 primer and 1X iCycler SYBRGREEN SuperMix (Bio-Rad, Hercules, CA). PCR primers (Invitrogen, Grand Island, NY) utilized to detect rat AdipoR1 in H9c2 myotubes and human AdipoR2 in HepG2 cells were as follows: rat AdipoR1 (forward: 5'-CTTCTACTGCTCCCCACAGC -3' and reverse: 5'-TCCCAGGAACACTCCTGCTC-3') and human AdipoR2 (forward: 5'-ATAGTCTCCCAGTGGGACATG-3' and reverse: 5'-AGGATCCGGGCAGCATACA -3'). The PCR cycling condition for rat AdipoR1 primer was carried out with an initial denaturing at 95°C for 3 min, followed by 40 cycles consisting of denaturing at 95°C for 30 sec, annealing at 60.7°C for 1 min, and extending at 72°C for 30 sec. For human AdipoR2 primer the annealing temperature was 58°C. After PCR, a Ct value was obtained using the software provided by the manufacturer. Identification the presence of AdipoR1/R2 receptors was accomplished by obtaining one peak on each melting curve

graph.

Measurement of glycogen synthesis

HepG2 cells were cultured in 24-well plates and serum starved overnight before experiments. Cells were treated with different concentrations of insulin and/or adiponectin and 0.5 μCi D-[^{14}C] glucose for 3 hours at 37°C. After the media was removed, 1.0 M KOH containing 1mg glycogen was added and the incubation was continued for another 1 h and 40 min at 60°C. Ice-cold ethanol was subsequently added to precipitate the glycogen. The precipitated glycogen was then separated by centrifugation at 3,600 g for 10 min and washed twice with ice-cold ethanol. Final precipitable radioactivity is solubilized in 0.2 N HCl and counted in a liquid scintillation counter.

Measurement of IRS-1 phosphorylation

HepG2 cells were cultured in 60-mm dishes and serum starved overnight before treated with insulin (10 nM), bacterially generated adiponectin (20 μg/ml), mammalian-cell expressed adiponectin (1 μg/ml) and the combination of insulin and adiponectin for 30 minutes. Then cells were lysed with RIPA buffer (1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.9% NaCl, 25 mM Tris, 1mM EDTA, pH=6.8) containing phosphatase and protease inhibitors.

Protein concentrations of these cell lysates were determined using Bio-Rad DC protein assay method as follows: 1.5 mg/ml, 0.8 mg/ml, 0.4 mg/ml, 0.2 mg/ml and 0.1 mg/ml of BSA were prepared from stock solution (15 mg/ml) as standard solutions. Reagent A1 was prepared by mixing reagent S and A (Bio-Rad, Hercules, CA). 5 µl of each standard and cell lysate sample, 25 µl of reagent A1 and 200 µl of reagent B were added into each well of a microtiter plate and incubated at room temperature for 15 min. All the samples and reagents were mixed thoroughly using a microplate mixer and the light absorbance of each sample was measured at 750 nm wavelength. Protein concentration of each cell lysate sample was also calculated based on the generated standard curve.

Anti-IRS-1 antibody was utilized to immunoprecipitate IRS-1 molecule from those cell lysates. Cell lysates were first diluted to a concentration of 1 µg/µl and transferred to microcentrifuge tubes. Anti-IRS-1 antibody was added into these tubes and the reaction mixture was gently rocked at 4°C overnight. The immunocomplex was captured by adding washed Protein A agarose bead slurry into the tubes and gently rocking the reaction mixture at 4°C for 2 h. The agarose beads were collected by brief (60 sec) centrifugation (1000 rpm), resuspended in 2X Laemmli buffer and boiled for 5 min. Supernatant fraction of the immunoprecipitation products were then subjected to western blotting analysis: 20 µl of the supernatants was separated by 7% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were then

blocked with 3% nonfat dry milk and incubated overnight with a primary antiphosphotyrosine antibody (4G10) (1:500 dilution), followed by incubation with a mouse anti-rabbit secondary antibody (1:500 dilution) for one and a half hours. Resultant bands were visualized by the BioRad Quantity One Flour-S Multi-Imager System to determine the amount of phosphorylated IRS-1 in each sample.

Measurement of glucose uptake

H9c2 myotubes were grown in a 24-well plate and serum starved overnight before experiments. The myotubes were washed twice with 500 µl warm modified PBS (137 mM NaCl, 2.7 mM KCl, 15.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 10 mM HEPES, pH= 7.4), then treated with/without insulin (10 nM), bacterially generated adiponectin (20 µg/ml and 30 µg/ml) and mammalian-cell generated adiponectin (1 µg/ml and 5 µg/ml) in 200 µl warm PBS for 20 min. After treatment, uptake of 10 µM 2-[^{3}H] deoxyglucose was measured over 10min. Reactions were terminated by the removal of the incubation media and rapid washing with ice-cold 20 µM phloretin. The myotubes were then dissolved in 500 µl of 0.1 N KOH, and aliquots were counted by liquid scintillation counting.

Statistical analysis

Results were expressed as means ± the standard error of the mean (SEM). Unpaired Student's *t* tests were performed to analyze the differences between various treatment groups. $p<0.05$ was considered significant for all analysis.

RESULTS

Effect of adiponectin on pyruvate-stimulated gluconeogenesis

Scherer *et al*. previously demonstrated that adiponectin enhanced hepatic insulin action by enhancing the suppression of hepatic glucose production (70). To evaluate if the eukaryotically expressed adiponectin has biological activity and works in our experimental system, we examined its effect on hepatic gluconeogenesis. Under basal conditions, the subphysiological concentration of insulin did not affect hepatic glucose production. Adiponectin did not have any effect on basal gluconeogenesis either. Pyruvate stimulated hepatic glucose production by about 20% compared to control. The subphysiological concentration of insulin did not have any effect on this pyruvate-stimulated gluconeogenesis. Importantly, the presence of adiponectin led to a 20% decrease in the pyruvate-stimulated gluconeogenesis, which almost completely reverted the increased glucose production to control levels (Figure 10).

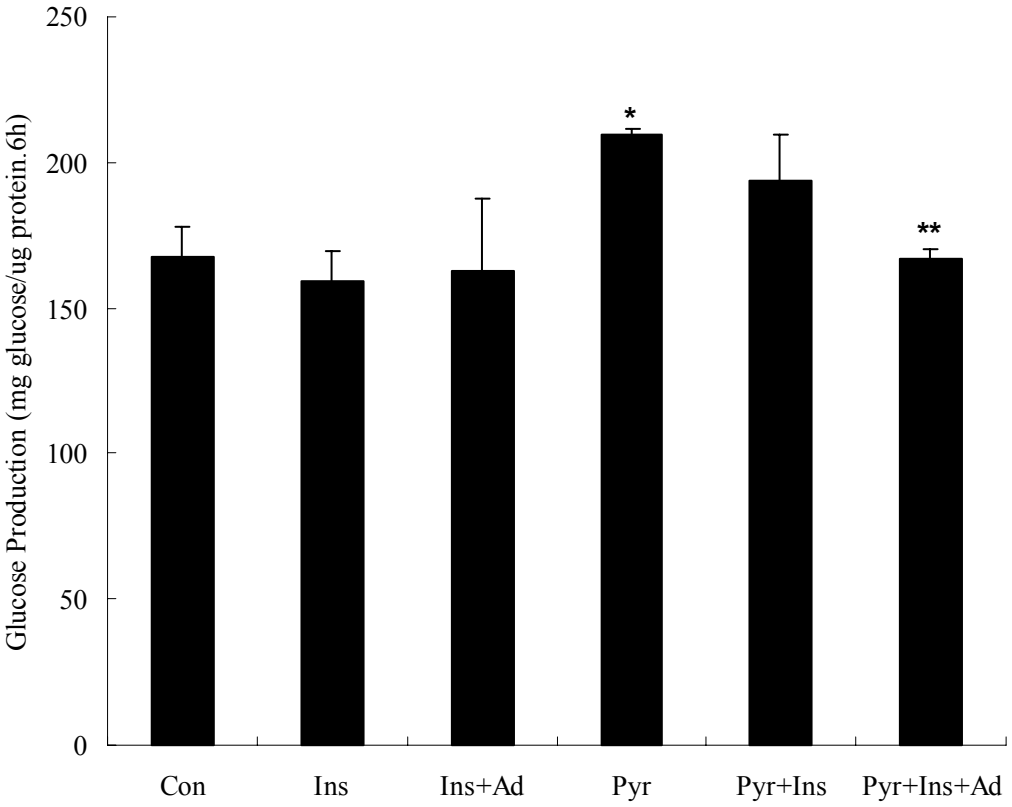


Figure 10: Eukaryotically expressed adiponectin inhibits pyruvate-stimulated gluconeogenesis in primary mouse hepatocytes. Primary mouse hepatocytes were incubated in complete RPMI 1640 medium with/without 33 pM insulin and/or 2 μg/ml of adiponectin for 24 h, glucose production was then measured by incubating these cells in glucose-free RPMI 1640 medium in the presence or absence of 5 mM pyruvate for another 6 h. The medium in each dish was collected and a glucose analysis kit was utilized to analyze the glucose concentration. Hepatic glucose production was calculated by measuring the amount of glucose produced in 6 hours per μg of hepatocyte lysate protein. Data are the means ± S.E.M., *p<0.05 vs control under basal conditions; **p<0.05 vs pyruvate-stimulated control. Abbreviation: Con: Control; Ins: Insulin; Pyr: Pyruvate; Ad: Adiponectin.

The presence of AdipoR2 receptor in HepG2 cells

Our initial studies in primary mouse hepatocytes demonstrated an effect of adiponectin on hepatic glucose metabolism. In order to further characterize this important metabolic observation, we chose a hepatic cell culture model frequently used to study insulin signaling and glucose responsiveness. To determine the effect of adiponectin on hepatic glycogen synthesis as well as its signal transduction pathway in the liver, we used a human hepatoma cell line (HepG2) as an experimental model. Since adiponectin has two receptors, AdipoR1 and AdipoR2, with AdipoR2 predominantly expressed in liver (41), western blotting analysis was first performed to determine if HepG2 cells express the AdipoR2 receptor. Utilizing western blotting analysis, we determined that HepG2 cells have AdipoR2 receptor (Figure 11). This was later confirmed by another group using several hepatoma cell line models (76).

We also utilized Real-Time polymerase chain reaction (PCR) analysis to detect AdipoR2 gene expression in HepG2 cells. A relative Ct value of 18.68 was quantified for AdipoR2 gene expression in this cell line (Figure 12A). In the melt curve graph, only one peak was identified at around 87°C, which was the melting temperature of AdipoR2 (Figure 12B).

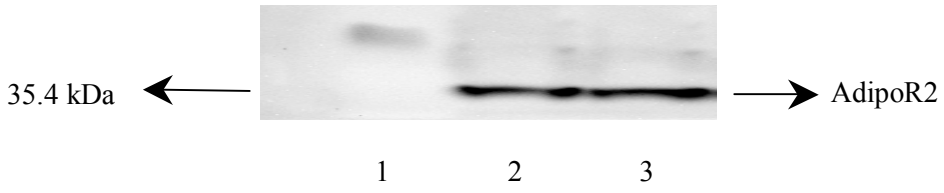


Figure 11: AdipoR2 receptor is present in HepG2 cells. Western blotting analysis: AdipoR2 antibody was used to confirm the presence of AdipoR2 (~35.4 kDa) in total membranes prepared from HepG2 cells. Lane1: standard marker; Lane 2 and 3: cell membrane samples.

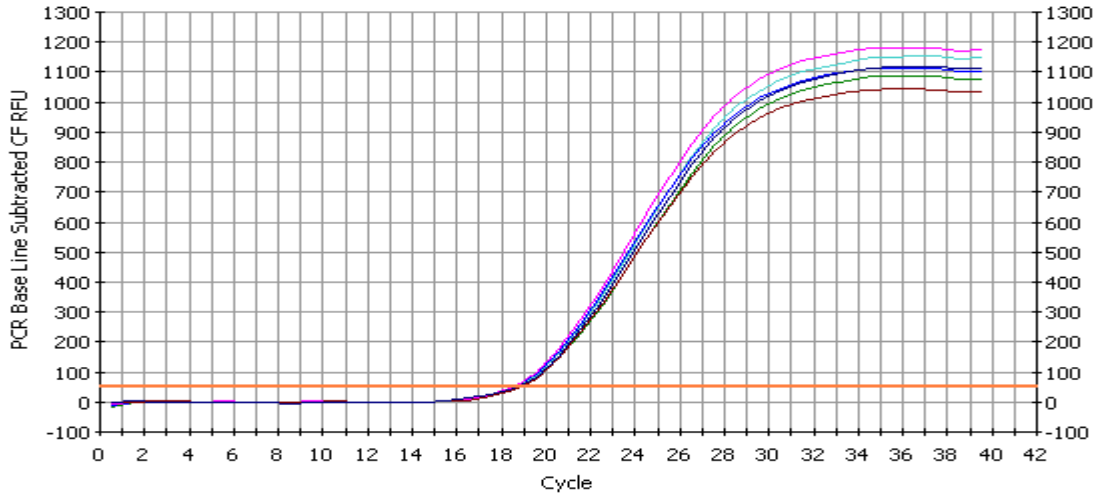


Figure 12A: AdipoR2 mRNA is expressed in HepG2 cells. Real-Time PCR base line quantification graph: a relative Ct value of 18.68 was quantified for AdipoR2 gene expression HepG2 cells.

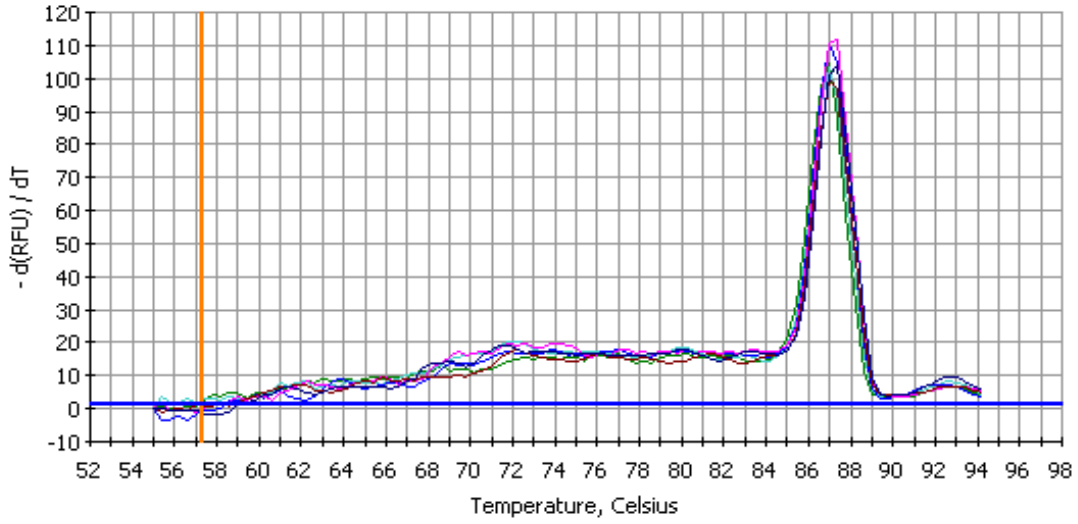


Figure 12B: AdipoR2 mRNA is expressed in HepG2 cells. Real-Time PCR melt curve graph: only one peak was identified at around 87°C, which was the melting temperature of AdipoR2.

Effect of insulin on AdipoR2 protein level in HepG2 cells

HepG2 cells are known to express high-affinity insulin receptors and insulin-like growth factor I and II (IGF-I and IGF-II) receptors. In our experiments, we determined the effect of different concentrations of insulin (1 nM, 10 nM and 100 nM) on AdipoR2 protein expression by western blotting analysis. Our results demonstrated that insulin significantly inhibited AdipoR2 protein expression in HepG2 cells in a concentration-dependent manner. For 10 nM and 100 nM of insulin, the inhibition rate was 54.96% and 30.82%, respectively. No significance was observed with 1 nM insulin (Figure 13).

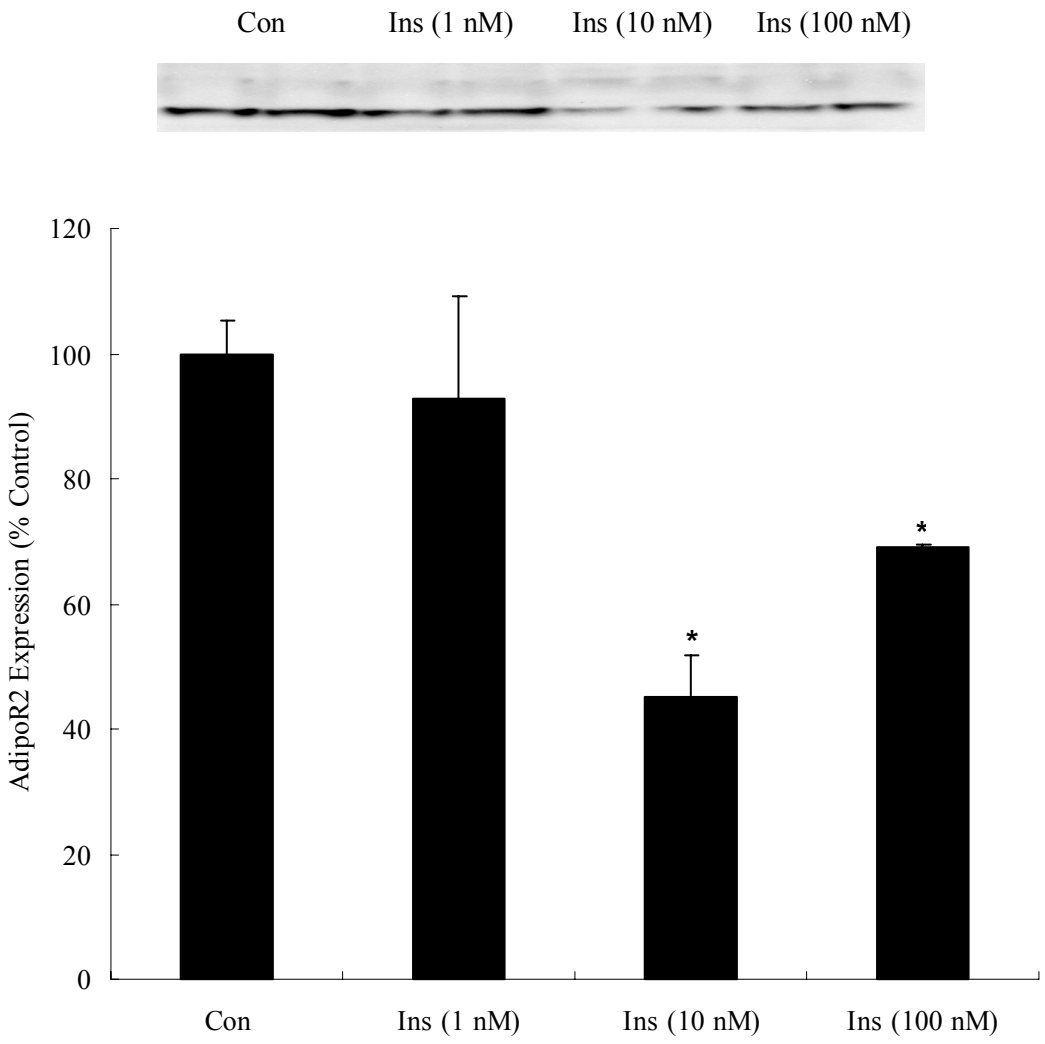


Figure 13: AdipoR2 protein expression is inhibited by insulin. HepG2 cells were treated with 1 nM, 10 nM and 100 nM insulin for 24 hours after serum-starved overnight. Cell membrane lysates were subjected to the same western blotting analysis as in Fig 11. Data are the means ± S.E.M for three independent experiments and are shown as % of control; *p<0.05 vs control. A representative blot is shown in the upper portion of the graph. Lane 1 and 2: control group; Lane 3 and 4: Insulin (1nM) group; Lane 5 and 6: Insulin (10 nM) group; Lane 7 and 8: Insulin (100 nM) group.

Effect of insulin on basal glycogen synthesis in HepG2 cells

Due to their insulin responsiveness, HepG2 cells are an excellent model system to study glycogen synthesis and insulin signaling, which can be verified by using this model to investigate the effect of insulin on hepatic basal glycogen synthesis. In this experiment, we treated HepG2 cells with 35 pM, 0.1 nM, 1 nM, 10 nM and 100 nM of insulin for 3 hours and glycogen synthesis during this period was evaluated. We demonstrated that insulin stimulates glycogen synthesis in a concentration dependent manner in HepG2 cells. 0.1 nM, 1 nM, 10 nM and 100 nM of insulin can stimulate glycogen synthesis by 21%, 32%, 48% and 58% respectively compared with the control group (Figure 14).

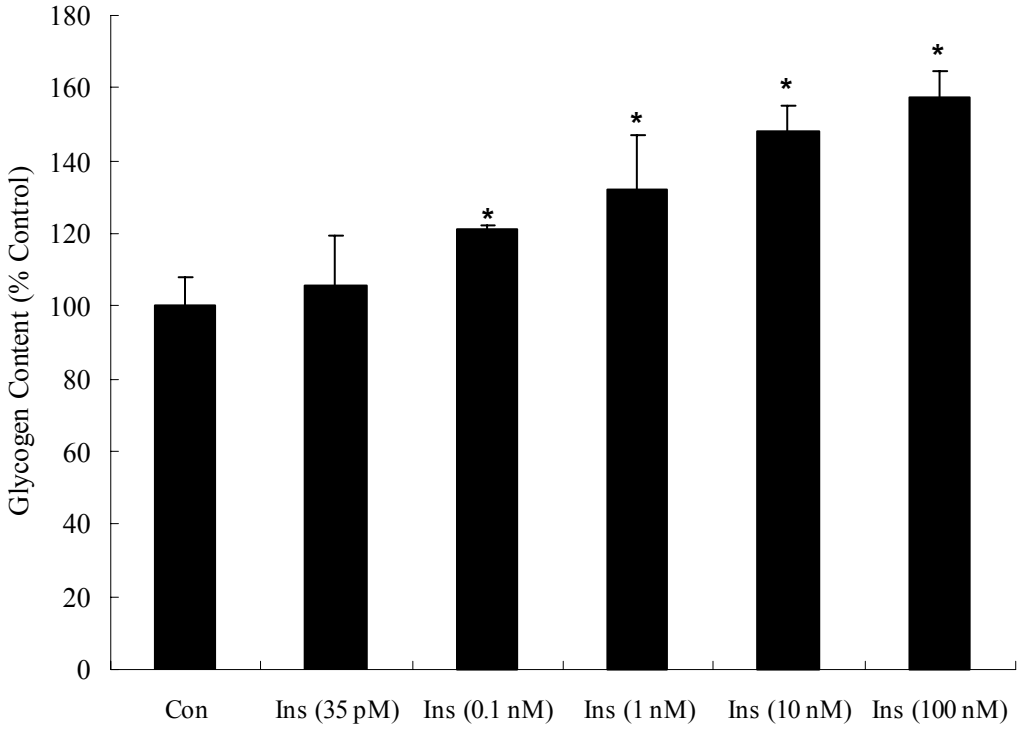


Figure 14: Insulin stimulates glycogen synthesis in HepG2 cells. HepG2 cells were serum starved overnight and then treated with different concentrations of insulin (35 pM, 0.1 nM, 1 nM, 10 nM and 100 nM) and 0.5 μCi D-[^{14}C] glucose for 3 hours at 37°C. Glycogen synthesis was then evaluated by counting the radioactivity incorporated into glycogen in a liquid scintillation counter. Data are the means ± S.E.M. and values are converted to percentages of control value (100%). *p<0.05 vs control. Abbreviation: Con: Control; Ins: Insulin.

Effect of adiponectin on basal and insulin-stimulated glycogen synthesis in HepG2 cells

Full-length adiponectin has been reported to decrease hepatic glucose production by inhibiting hepatic G-6-Pase and PEPCK mRNA expression (64). However, the effects of adiponectin on hepatic glycogen synthesis have not been determined. In our studies, we first examined the effects of both bacterially generated and eukaryotically expressed adiponectin on basal hepatic glycogen synthesis. HepG2 cells were serum starved overnight and treated with different concentrations of bacterially generated adiponectin (20 μg/ml and 30 μg/ml) or eukaryotically expressed adiponectin (1 μg/ml and 5 μg/ml) together with 0.5 μCi D-[^{14}C] glucose for 3 hours at 37°C. Here we utilized physiological concentrations of eukaryotically expressed adiponectin. Since bacterially generated adiponectin may contain fewer biological active forms than eukaryotically expressed one, we utilized higher concentrations of bacterially generated adiponectin. Glycogen synthesis was calculated in an identical fashion as in the insulin-stimulation experiment. Bacterially generated adiponectin did not have any effects on hepatic basal glycogen synthesis in HepG2 cells, while eukaryotically expressed adiponectin significantly inhibited hepatic glycogen synthesis (Figure 15). For 1 μg/ml eukaryotically expressed adiponectin, the inhibition rate was about 37% compared with control; 5 μg/ml of eukaryotically expressed adiponectin inhibited hepatic glycogen synthesis about 28% compared with control. Our result demonstrated that only eukaryotically expressed but not bacterially expressed adiponectin can affect basal glycogen synthesis in liver.

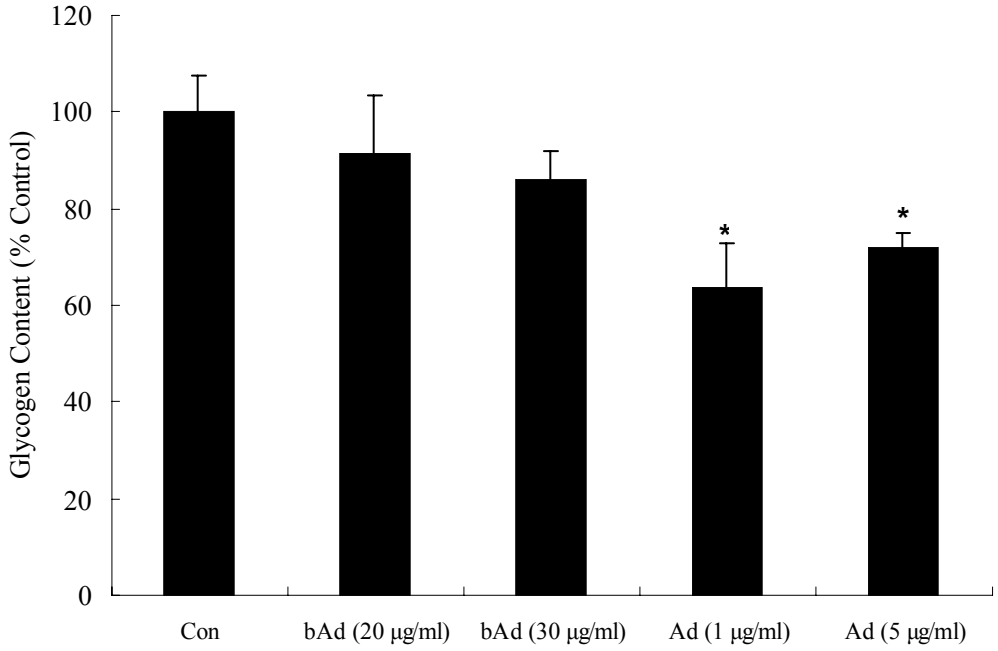


Figure 15: Eukaryotically expressed adiponectin inhibits basal glycogen synthesis in HepG2 cells. HepG2 cells were serum starved overnight and treated with different concentrations of bacterially generated adiponectin (20 μg/ml and 30 μg/ml) or eukaryotically expressed adiponectin (1 μg/ml and 5 μg/ml) together with 0.5 μCi D-[^{14}C] glucose for 3 hours at 37°C. Glycogen synthesis was calculated in the same way as in Fig 14. Both 1 μg/ml and 5 μg/ml of eukaryotically expressed adiponectin significantly inhibited basal glycogen synthesis, however, neither concentrations of bacterially generated adiponectin have any apparent effect on hepatic glycogen synthesis. Data are the means ± S.E.M. and values are converted to percentages of control value (100%). *p<0.05 vs control group. Abbreviation: Con: Control; bAd: Bacterially generated adiponectin; Ad: Eukaryotically expressed adiponectin.

After demonstrating that eukaryotically expressed adiponectin inhibits basal glycogen synthesis in HepG2 cells, we further examined if this protein has any effect on insulin-stimulated hepatic glycogen synthesis. HepG2 cells were treated with or without 1 nM insulin or 1 nM insulin plus eukaryotically expressed adiponectin (1 μg/ml and 5 μg/ml) together with 0.5 μCi D-[^{14}C] glucose for 3 hours and glycogen synthesis was evaluated during this process. Figure 16 showed that 1 nM insulin significantly stimulated glycogen synthesis compared with control group. 5 μg/ml of eukaryotically expressed adiponectin inhibited this insulin-stimulated glycogen synthesis for about 44.7%. This demonstrated that eukaryotically expressed adiponectin also inhibits insulin-stimulated hepatic glycogen synthesis.

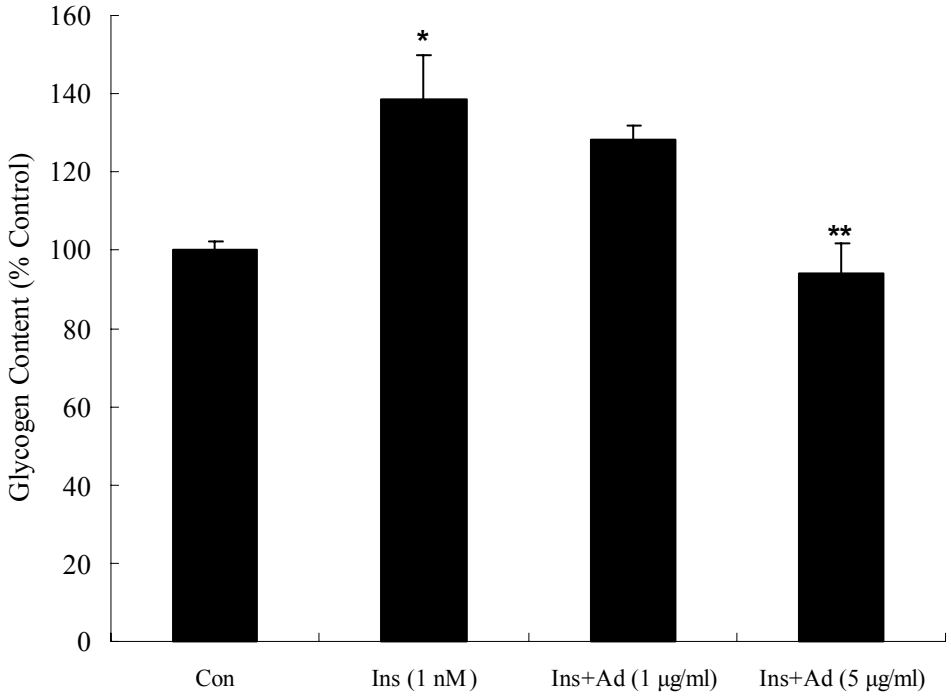


Figure 16: Eukaryotically expressed adiponectin inhibits insulin-stimulated glycogen synthesis in HepG2 cells. HepG2 cells were serum starved overnight and treated with or without 1 nM insulin, 1 nM insulin plus eukaryotically expressed adiponectin (1 μg/ml and 5 μg/ml) together with 0.5 μCi D-[^{14}C] glucose for 3 hours and glycogen synthesis was evaluated during this process. 5 μg/ml of eukaryotically expressed adiponectin was found to inhibit insulin-stimulated glycogen synthesis significantly. Data are the means ± S.E.M. and values are converted to percentages of control value (100%). *p<0.05 vs control group. **p<0.05 vs insulin-stimulated control group. Abbreviation: Con: Control; Ins: Insulin; Ad: Eukaryotically expressed adiponectin.

Effects of insulin and adiponectin on IRS-1 phosphorylation in HepG2 cells

Since eukaryotically expressed adiponectin can inhibit basal and insulin-stimulated glycogen synthesis in liver, we next determined the mechanism involved in this process. Adiponectin may work through the insulin signaling pathway to activate the IRS-1 and PI-3 Kinase, therefore increasing hepatic insulin sensitivity (72). In our experiments, we examined if adiponectin can phosphorylate IRS-1 by using both immunoprecipitation and western blotting analysis. Our data demonstrates that IRS-1 is not phosphorylated by either bacterially generated or eukaryotically expressed adiponectin in HepG2 cells (Figure 17). However, as a positive control, phosphorylation of IRS-1 is observed in the presence of insulin (10 nM). Therefore, adiponectin does not signal through the traditional insulin signaling pathway to inhibit hepatic glycogen synthesis.

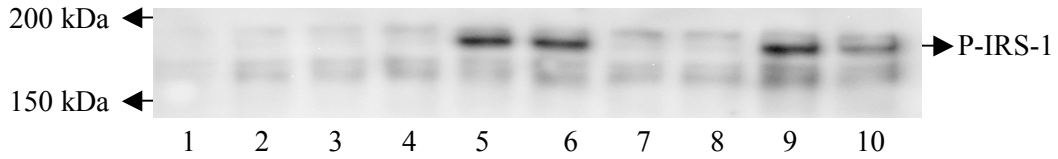


Figure 17: IRS-1 is not phosphorylated by adiponectin in HepG2 cells. HepG2 cells were serum starved overnight before treating them with insulin (10 nM), bacterially generated adiponectin (20 μg/ml), eukaryotically expressed adiponectin (1 μg/ml) and the combination of insulin and adiponectin for 30 min. Then cells were lysed and IRS-1 antibody was utilized to immunoprecipitate IRS-1 molecule. The immunoprecipitation products were subjected to western blotting analysis using antiphosphotyrosine antibody (4G10) as the primary antibody to determine the level of phosphorylated IRS-1 in each group. 10 nM of insulin phosphorylates IRS-1 in HepG2 cells while neither bacterially generated adiponectin nor eukaryotically expressed protein phosphorylates IRS-1 directly. Lane1 and 2: Control; Lane 3 and 4: Bacterially generated adiponectin; Lane 5 and 6: Insulin; Lane 7 and 8: Eukaryotically expressed adiponectin; Lane 9 and 10: Insulin plus eukaryotically expressed adiponectin treated group.

The presence of AdipoR1 receptor in H9c2 myotubes

We have demonstrated that eukaryotically expressed adiponectin inhibits basal and insulin-stimulated glycogen synthesis in HepG2 cells. We next want to examine the effects of adiponectin on muscle basal glucose uptake. A rat ventricular cell line H9c2 with many properties of skeletal muscle was utilized in this study. We first determined if H9c2 myotubes express AdipoR1 receptor.

Here we only utilized Real-Time PCR analysis to detect AdipoR1 gene expression in H9c2 myotubes. A relative Ct value of 21.63 was quantified for AdipoR1 gene expression (Figure 18A) and only one peak was identified at around 87.5°C in the melt curve, which was the melting temperature of AdipoR1 (Figure 18B).

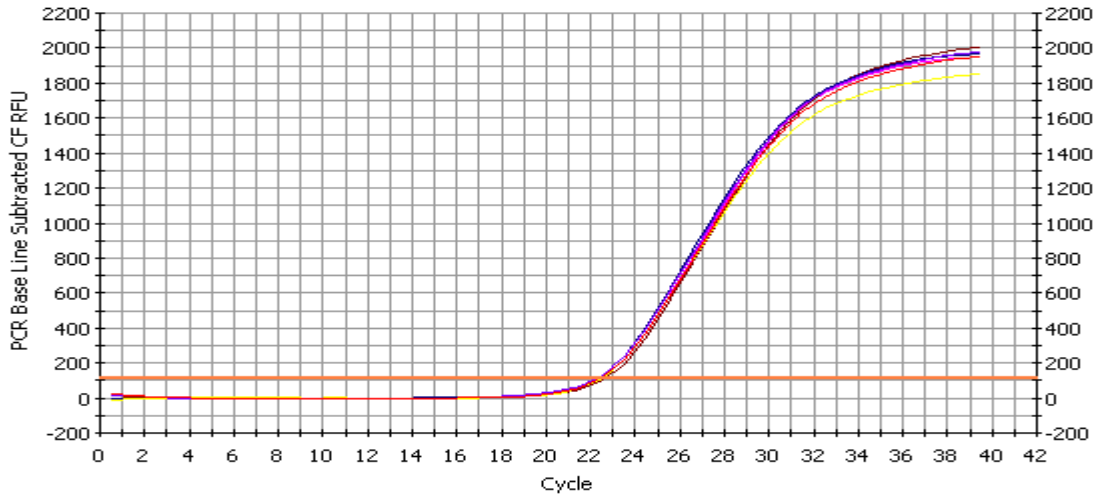


Figure 18A: AdipoR1 mRNA is expressed in H9c2 myotubes. Real-Time PCR base line quantification graph: a relative Ct value of 21.63 was quantified for AdipoR1 gene expression H9c2 myotubes.

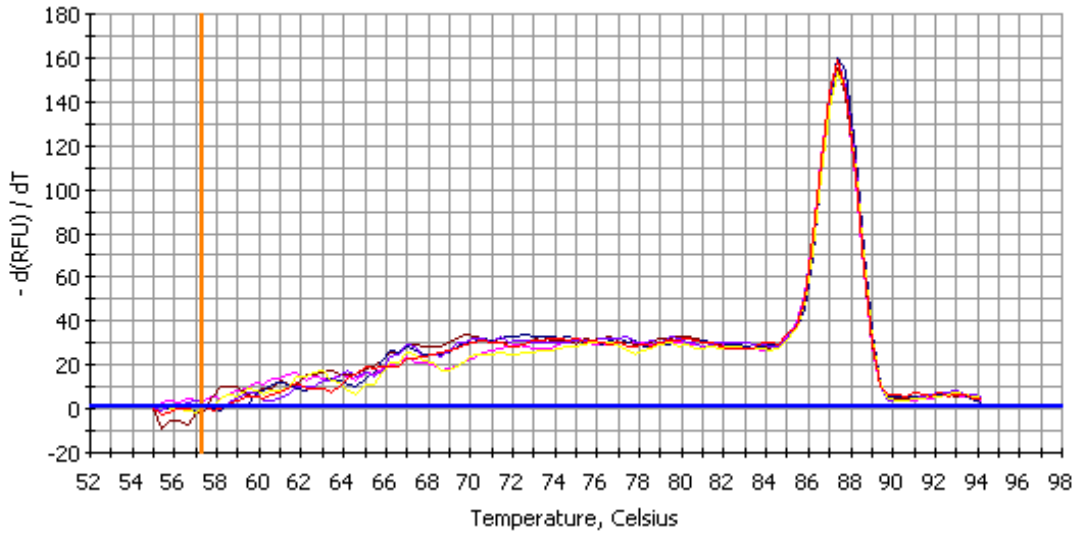


Figure 18B: AdipoR1 mRNA is expressed in H9c2 myotubes. Real-time PCR melt curve graph: only one peak was identified at around 87.5°C, which was the melting temperature of AdipoR1.

Effect of adiponectin on basal glucose uptake in H9c2 cells

Globular adiponectin has recently been shown to increase glucose uptake in skeletal muscle cells via GLUT4 translocation (74). After we proved that AdipoR1 receptor is present in H9c2 cells by Real-Time PCR, we further examined the effects of adiponectin on basal glucose uptake in this cell line. Cells were treated with or without 10 nM of insulin, different concentrations of bacterially generated (20 μg/ml and 30μg/ml) and eukaryotically expressed (1μg/ml and 5μg/ml)adiponectin for about 20 min, then uptake of 2-[^{3}H] deoxyglucose was measured over 10min and radioactivity in myotube lysates was counted by liquid scintillation counting. Our experimental result (Figure 19) showed that 10 nM of insulin, 20 μg/ml and 30 μg/ml of bacterially expressed adiponectin stimulate basal glucose uptake for about 2.5 fold, 105% and 92% respectively compared with control group, while eukaryotically expressed adiponectin does not have any apparent effect on basal glucose uptake in H9c2 cells. This demonstrated that only bacterially generated adiponectin but not eukaryotically expressed protein can stimulate basal glucose uptake in H9c2 cells.

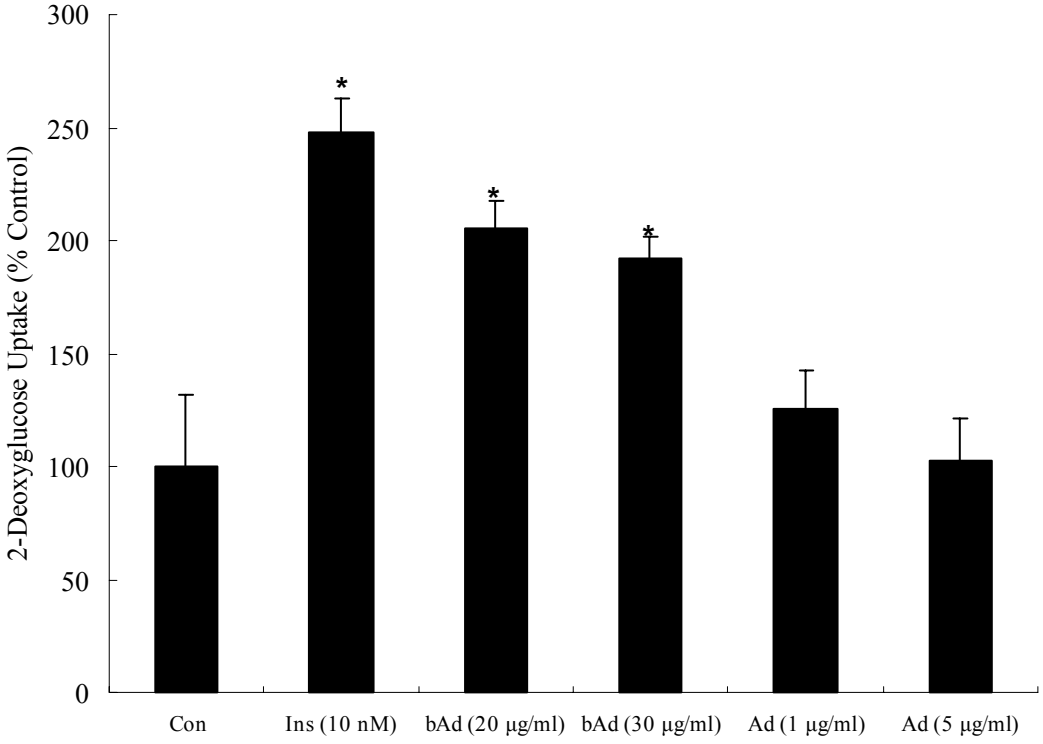


Figure 19: Bacterially expressed adiponectin stimulates basal glucose uptake in H9c2 cells. H9c2 myotubes were grown in a 24-well plate and serum starved overnight before experiments. The myotubes were washed twice with 500 μl warm modified PBS and then treated with/without insulin (10 nm), bacterially generated adiponectin (20 μg/ml and 30 μg/ml) and eukaryotically expressed adiponectin (1 μg/ml and 5 μg/ml) in 200 μl warm PBS for 20 min. After treatment, uptake of 10 μM 2-[^{3}H] deoxyglucose was measured over 10 min. Reactions were then terminated, myotubes were lysed and aliquots were counted by liquid scintillation counting. Like insulin, both concentrations of bacterially expressed adiponectin significantly stimulated basal glucose uptake in H9c2 cells, while eukaryotically expressed adiponectin did not have any apparent effect on basal glucose uptake in H9c2 cells. Data are the means ± S.E.M. and values are converted to percentages of control value (100%). *p<0.05 vs control group. Abbreviation: Con: Control; Ins: Insulin; bAd: Bacterially generated adiponectin; Ad: Eukaryotically expressed adiponectin.

Western blotting image of eukaryotically expressed adiponectin and bacterially expressed adiponectin

Our studies have demonstrated that eukaryotically expressed adiponectin can affect hepatic glycogen synthesis while bacterially expressed adiponectin does not have this effect. Also, bacterially expressed adiponectin affects muscle glucose uptake, but eukaryotically expressed adiponectin does not. We next determined if there is any difference between the multimer formations of these two sources of adiponectin. The same amount of HEK293 cell-generated and bacterially expressed mouse adiponectin was subjected to western blotting analysis using mouse adiponectin antibody as primary antibody. Samples were analyzed under normal, heating, reducing conditions as well as the combination of heating and reducing conditions. The image (Figure 20) shows monomer and dimer formations of the protein. Under normal conditions (Lane 1 and 3), both sources of protein contain very similar amount of monomer and dimer. Heating and reducing (Lane 2 and 6) make all the multimers larger than a monomer convert into monomers, the amount of which is higher in denatured eukaryotically expressed adiponectin than in denatured bacterially expressed adiponectin. This suggests that the amount of higher molecular weight form of adiponectin may be higher in eukaryotically expressed adiponectin than in bacterially expressed adiponectin.

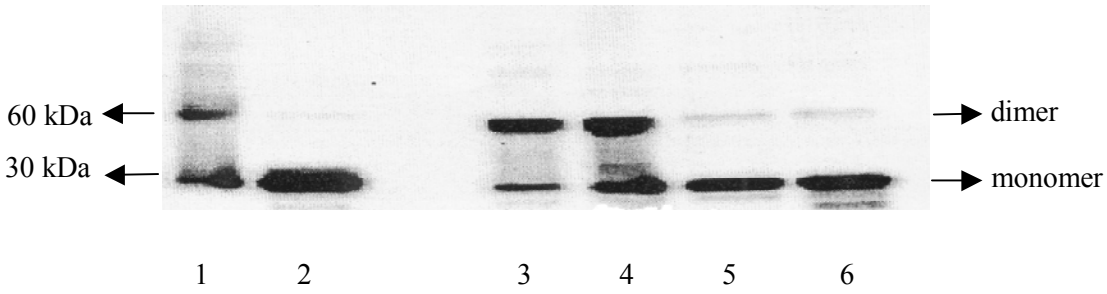


Figure 20: Western blotting image of eukaryotically expressed and bacterially expressed adiponectin. HEK293 cell-generated and bacterially expressed mouse adiponectin were subjected to 10% SDS-PAGE with or without 100°C heat denaturation for 5 min, and with or without reduction by 5% 2-mercaptoethanol. Adiponectin was detected by anti-mouse adiponectin antibody. Lane 1 and 3 show the monomer and dimer formations of both sources of adiponectin under normal conditions (High molecular weight forms can not be shown here). Both were converted to monomers when heat-denatured and reduced (Lane 2 and 6). Bacterially expressed adiponectin was converted to monomers and dimers under heating conditions (Lane 4), to monomers when reduced (Lane 5) [This figure was obtained from Qiao Zhong with permission].

DISCUSSION

Adiponectin is a 30 kDa protein secreted exclusively by adipose tissue under normal conditions. It is a metabolic hormone whose serum level is decreased in obesity and type 2 diabetes mellitus. Two adiponectin receptors that regulate its anti-diabetic effect have recently been cloned in mice, AdipoR1 and AdipoR2. AdipoR1 is ubiquitously and most abundantly expressed in muscle, whereas AdipoR2 is most abundantly expressed in liver. These two receptors are capable of mediating increased AMPK and PPARα ligand activities, as well as fatty-acid oxidation and glucose uptake by adiponectin.

In the present study, we utilized two approaches to demonstrate that HepG2 cells express both AdipoR2 protein and mRNA. We subsequently investigated the effects of two sources of adiponectin on hepatic glycogen sysnthesis as well as the mechanisms involved in this process. We also demonstrated that H9c2 cells express AdipoR1 mRNA, which enabled us to study and compare the effects of those two sources of adiponectin on muscle glucose uptake. In addition, we did some preliminary studies on the regulation of AdipoR2 expression, which suggested that HepG2 cells might be a good model to study adiponectin receptors in the future.

Our study demonstrated for the first time that eukaryotically, but not bacterially expressed adiponectin inhibits hepatic basal glycogen synthesis, which suggested that these two sources of adiponectin contain different multimers. SDS-PAGE image of these proteins under reducing and heating conditions demonstrated that eukaryotically expressed adiponectin might contain more higher molecular weight forms than bacterially produced adiponectin, which is very similar to some previous findings. Because bacteria are unable to process the hydroxyl-prolinated collagenous domain and allow for proper folding and orientation of the disulfide bond within this domain, the bacterially produced adiponectin is secreted as lower order forms (77). Scherer *et al.* suggested that adiponectin's effect on hepatic insulin sensitivity is highly dependent on the presence of higher order structures, such as hexamers and HMW forms (29). Importantly, some previous studies showed that the hepatic effects of adiponectin require hydroxylation and glycosylation of residues within the collagenous domain of adiponectin (28), which may also explain why the bacterially produced adiponectin lacking post-translational modifications in the collagenous domain does not affect hepatic glucose metabolism.

We have demonstrated that eukaryotically expressed adiponectin inhibits pyruvate-stimulated gluconeogenesis in primary mouse hepatocytes, which is similar to Scherer *et al* (70). Adiponectin can enhance the inhibition effect of insulin on hepatic gluconeogenesis, which shows the insulin-sensitizing property of this protein. In our study, we proved that eukaryotically expressed adiponectin also inhibits

insulin-stimulated glycogen synthesis in liver, which suggested that adiponectin might play a major role in inhibiting hepatic glycogen synthesis, therefore providing more glucose for glycolysis and ATP generation in liver.

We further investigated the mechanisms involved in the effects of adiponectin on hepatic glycogen synthesis. This may also help to investigate the whole hepatic signal transduction pathway of adiponectin in the future. There are two pathways through which eukaryotically expressed adiponectin may work to inhibit hepatic glycogen synthesis: First it may work through the insulin-signaling pathway; also it may stimulate AMPK activity, phosphorylate and inactivate glycogen synthase, thus inhibiting glycogen synthesis. Our experimental result showed that insulin phosphorylates IRS-1 in HepG2 cells, while neither bacterially generated nor eukaryotically expressed protein phosphorylates IRS-1 directly. So adiponectin does not work through insulin signal pathway to inhibit basal glycogen synthesis. We then examined if eukaryotically expressed adiponectin can increase AMPK phosphorylation. Our western blotting data (not shown) appear to show that this protein did not increase the phosphorylation of AMPK in HepG2 cells either. Because we did not see the phosphorylation of AMPK by AICAR (5'-phosphoribosyl-5-aminoimidazole-4-carboxamide), an AMPK activator, in HepG2 cells, we could not exclude the possibility that our experimental conditions or process masked the stimulation effect. Since adiponectin has anti-tumor function, it might suppress the growth and energy metabolism of HepG2 cells, and the effects of

adiponectin on phosphorylation of AMPK might be concealed.

Scherer *et al.* found that the effects of adiponectin on muscle only occur with the bacterially produced full-length or the globular domain of adiponectin (29). In our studies, we demonstrated that bacterially but not eukaryotically expressed adiponectin stimulates basal glucose uptake in the muscle, which is very similar to their finding. Also Sweeney *et al.* found that globular adiponectin increases GLUT4 translocation and glucose uptake but reduces glycogen synthesis in rat skeletal muscle cells (74), which suggested a similar effect of globular adiponectin in muscle. Whether this effect is purely pharmacological or a reflection of a physiological aspect of adiponectin function has not been decided yet, as forming a multimer of higher molecular weight is very important for its bioactivity in liver but not in muscle. We have identified that AdipoR1 mRNA is present in H9c2 cells by realtime PCR. AdipoR1 is reported to be expressed predominantly in skeletal muscle and exhibits a higher affinity for globular adiponectin (41). Since bacterially expressed adiponectin is secreted as lower order forms, it might contain globular adiponectin as well. Differences in binding affinity of bacterially and eukaryotically expressed adiponectin in muscle cells might also be able to explain its different effect on muscle.

The present study verified the presence of AdipoR1 mRNA in H9c2 cells and AdipoR2 in HepG2 cells for the first time. In addition, insulin was found to inhibit AdipoR2 protein expression in HepG2 cells. Kadowaki *et al.* suggested that insulin may also be a negative regulator to AdipoR2 mRNA level (49). This implied that AdipoR2

might also be involved in the metabolic effect of adiponectin. However, some other groups suggested that AdipoR1, but not AdipoR2 is involved in glucose or lipid metabolism (50-53). Our realtime-PCR data (not shown) appear to demonstrate that insulin does not affect AdipoR2 mRNA expression in HepG2 cells. There might be some post-translational effect of insulin on AdipoR2 expression.

SUMMARY

- Eukaryotically expressed adiponectin inhibits pyruvate-stimulated gluconeogenesis in primary mouse hepatocytes.
- AdipoR2 protein and mRNA are expressed in HepG2 cells.
- Insulin inhibits AdipoR2 protein expression in HepG2 cells.
- Eukaryotically but not bacterially expressed adiponectin inhibits basal and insulin-stimulated glycogen synthesis in HepG2 cells.
- IRS-1 is not phosphorylated by adiponectin in HepG2 cells.
- AdipoR1 mRNA is expressed in H9c2 cells.
- Bacterially but not eukaryotically expressed adiponectin stimulates basal glucose uptake in H9c2 cells.
- Eukaryotically expressed adiponectin contains more higher molecular weight forms than bacterially expressed adiponectin.

BIBLIOGRAPHY

1. McMurry and Castellion: Carbohydrate Metabolism. 95 A.D.,

2. Stumvoll M, Perriello G, Meyer C, Gerich J: Role of glutamine in human carbohydrate metabolism in kidney and other tissues. *Kidney Int.* 55:778-792, 1999

3. Bonadonna RC: Alterations of glucose metabolism in type 2 diabetes mellitus. An overview. *Rev.Endocr.Metab Disord.* 5:89-97, 2004

4. Kadowaki T, Hara K, Yamauchi T, Terauchi Y, Tobe K, Nagai R: Molecular mechanism of insulin resistance and obesity. *Exp.Biol.Med.(Maywood.)* 228:1111-1117, 2003

5. Haque WA, Garg A: Adipocyte biology and adipocytokines. *Clin.Lab Med.* 24:217-234, 2004

6. Abate N, Garg A: Heterogeneity in adipose tissue metabolism: causes, implications and management of regional adiposity. *Prog.Lipid Res.* 34:53-70, 1995

7. Fruhbeck G, Gomez-Ambrosi J, Muruzabal FJ, Burrell MA: The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am.J.Physiol Endocrinol.Metab* 280:E827-E847, 2001

8. Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H, Watanabe E, Takagi T, Akiyoshi M, Ohtsubo T, Kihara S, Yamashita S, Makishima M, Funahashi T, Yamanaka S, Hiramatsu R, Matsuzawa Y, Shimomura I: Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* 307:426-430, 2005

9. Garg A: Lipodystrophies. *Am.J.Med.* 108:143-152, 2000

10. Ouchi N, Kihara S, Funahashi T, Matsuzawa Y, Walsh K: Obesity, adiponectin and vascular inflammatory disease. *Curr.Opin.Lipidol.* 14:561-566, 2003

11. Stefan N, Stumvoll M, Vozarova B, Weyer C, Funahashi T, Matsuzawa Y, Bogardus C, Tataranni PA: Plasma adiponectin and endogenous glucose production in humans. *Diabetes Care* 26:3315-3319, 2003

12. Tsuchida A, Yamauchi T, Kadowaki T: Nuclear receptors as targets for drug development: molecular mechanisms for regulation of obesity and insulin resistance by peroxisome proliferator-activated receptor gamma, CREB-binding

protein, and adiponectin. *J.Pharmacol.Sci.* 97:164-170, 2005

13. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem.Biophys.Res.Commun.* 257:79-83, 1999

14. Hu E, Liang P, Spiegelman BM: AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J.Biol.Chem.* 271:10697-10703, 1996

15. Arita Y, Kihara S, Ouchi N, Maeda K, Kuriyama H, Okamoto Y, Kumada M, Hotta K, Nishida M, Takahashi M, Nakamura T, Shimomura I, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y: Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation* 105:2893-2898, 2002

16. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF: A novel serum protein similar to C1q, produced exclusively in adipocytes. *J.Biol.Chem.* 270:26746-26749, 1995

17. Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K: cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochem.Biophys.Res.Commun.* 221:286-289, 1996

18. Nakano Y, Tobe T, Choi-Miura NH, Mazda T, Tomita M: Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. *J.Biochem.(Tokyo)* 120:803-812, 1996

19. Funahashi T, Nakamura T, Shimomura I, Maeda K, Kuriyama H, Takahashi M, Arita Y, Kihara S, Matsuzawa Y: Role of adipocytokines on the pathogenesis of atherosclerosis in visceral obesity. *Intern.Med.* 38:202-206, 1999

20. Vasseur F, Lepretre F, Lacquemant C, Froguel P: The genetics of adiponectin. *Curr.Diab.Rep.* 3:151-158, 2003

21. Comuzzie AG, Funahashi T, Sonnenberg G, Martin LJ, Jacob HJ, Black AE, Maas D, Takahashi M, Kihara S, Tanaka S, Matsuzawa Y, Blangero J, Cohen D, Kissebah A: The genetic basis of plasma variation in adiponectin, a global endophenotype for obesity and the metabolic syndrome. *J.Clin.Endocrinol.Metab* 86:4321-4325, 2001

22. Beltowski J: Adiponectin and resistin--new hormones of white adipose tissue. *Med.Sci.Monit.* 9:RA55-RA61, 2003

23. Tsao TS, Murrey HE, Hug C, Lee DH, Lodish HF: Oligomerization state-dependent activation of NF-kappa B signaling pathway by adipocyte complement-related protein of 30 kDa (Acrp30). *J.Biol.Chem.* 277:29359-29362, 2002

24. Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M, Scherer PE: Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. *J.Biol.Chem.* 278:9073-9085, 2003

25. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, Vasseur F, Froguel P, Kimura S, Nagai R, Kadowaki T: Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin. *J.Biol.Chem.* 278:40352-40363, 2003

26. Tsao TS, Tomas E, Murrey HE, Hug C, Lee DH, Ruderman NB, Heuser JE, Lodish HF: Role of disulfide bonds in Acrp30/adiponectin structure and signaling specificity. Different oligomers activate different signal transduction pathways. *J.Biol.Chem.* 278:50810-50817, 2003

27. Kobayashi H, Ouchi N, Kihara S, Walsh K, Kumada M, Abe Y, Funahashi T, Matsuzawa Y: Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin. *Circ.Res.* 94:e27-e31, 2004

28. Wang Y, Xu A, Knight C, Xu LY, Cooper GJ: Hydroxylation and glycosylation of the four conserved lysine residues in the collagenous domain of adiponectin. Potential role in the modulation of its insulin-sensitizing activity. *J.Biol.Chem.* 277:19521-19529, 2002

29. Trujillo ME, Scherer PE: Adiponectin--journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. *J.Intern.Med.* 257:167-175, 2005

30. Yoda-Murakami M, Taniguchi M, Takahashi K, Kawamata S, Saito K, Choi-Miura NH, Tomita M: Change in expression of GBP28/adiponectin in carbon tetrachloride-administrated mouse liver. *Biochem.Biophys.Res.Commun.* 285:372-377, 2001

31. Berner HS, Lyngstadaas SP, Spahr A, Monjo M, Thommesen L, Drevon CA, Syversen U, Reseland JE: Adiponectin and its receptors are expressed in bone-forming cells. *Bone* 35:842-849, 2004

32. Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J, Paschke R: Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1

adipocytes. *Biochem.Biophys.Res.Commun.* 301:1045-1050, 2003

33. Moore GB, Chapman H, Holder JC, Lister CA, Piercy V, Smith SA, Clapham JC: Differential regulation of adipocytokine mRNAs by rosiglitazone in db/db mice. *Biochem.Biophys.Res.Commun.* 286:735-741, 2001

34. Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y: PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* 50:2094-2099, 2001

35. Kappes A, Loffler G: Influences of ionomycin, dibutyryl-cycloAMP and tumour necrosis factor-alpha on intracellular amount and secretion of apM1 in differentiating primary human preadipocytes. *Horm.Metab Res.* 32:548-554, 2000

36. Fasshauer M, Klein J, Neumann S, Eszlinger M, Paschke R: Adiponectin gene expression is inhibited by beta-adrenergic stimulation via protein kinase A in 3T3-L1 adipocytes. *FEBS Lett.* 507:142-146, 2001

37. Soares AF, Guichardant M, Cozzone D, Bernoud-Hubac N, Bouzaidi-Tiali N, Lagarde M, Geloen A: Effects of oxidative stress on adiponectin secretion and lactate production in 3T3-L1 adipocytes. *Free Radic.Biol.Med.* 38:882-889, 2005

38. Nishizawa H, Shimomura I, Kishida K, Maeda N, Kuriyama H, Nagaretani H, Matsuda M, Kondo H, Furuyama N, Kihara S, Nakamura T, Tochino Y, Funahashi T, Matsuzawa Y: Androgens decrease plasma adiponectin, an insulin-sensitizing adipocyte-derived protein. *Diabetes* 51:2734-2741, 2002

39. Xu A, Chan KW, Hoo RL, Wang Y, Tan KC, Zhang J, Chen B, Lam MC, Tse C, Cooper GJ, Lam KS: Testosterone selectively reduces the high molecular weight form of adiponectin by inhibiting its secretion from adipocytes. *J.Biol.Chem.* 2005

40. Meier U, Gressner AM: Endocrine regulation of energy metabolism: review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin. *Clin.Chem.* 50:1511-1525, 2004

41. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T: Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423:762-769, 2003

42. Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS, Lodish HF: T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. *Proc.Natl.Acad.Sci.U.S.A* 101:10308-10313, 2004

43. Kemler R: Classical cadherins. *Semin.Cell Biol.* 3:149-155, 1992

44. Ivanov DB, Philippova MP, Tkachuk VA: Structure and functions of classical cadherins. *Biochemistry (Mosc.)* 66:1174-1186, 2001

45. Kharroubi I, Rasschaert J, Eizirik DL, Cnop M: Expression of adiponectin receptors in pancreatic beta cells. *Biochem.Biophys.Res.Commun.* 312:1118-1122, 2003

46. Bluher M, Fasshauer M, Kralisch S, Schon MR, Krohn K, Paschke R: Regulation of adiponectin receptor R1 and R2 gene expression in adipocytes of C57BL/6 mice. *Biochem.Biophys.Res.Commun.* 329:1127-1132, 2005

47. Fasshauer M, Klein J, Kralisch S, Klier M, Lossner U, Bluher M, Paschke R: Growth hormone is a positive regulator of adiponectin receptor 2 in 3T3-L1 adipocytes. *FEBS Lett.* 558:27-32, 2004

48. Lord E, Ledoux S, Murphy BD, Beaudry D, Palin MF: Expression of adiponectin and its receptors in swine. *J.Anim Sci.* 83:565-578, 2005

49. Tsuchida A, Yamauchi T, Ito Y, Hada Y, Maki T, Takekawa S, Kamon J, Kobayashi M, Suzuki R, Hara K, Kubota N, Terauchi Y, Froguel P, Nakae J, Kasuga M, Accili D, Tobe K, Ueki K, Nagai R, Kadowaki T: Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin

sensitivity. *J.Biol.Chem.* 279:30817-30822, 2004

50. Inukai K, Nakashima Y, Watanabe M, Takata N, Sawa T, Kurihara S, Awata T, Katayama S: Regulation of adiponectin receptor gene expression in diabetic mice. *Am.J.Physiol Endocrinol.Metab* 2004

51. Wang H, Zhang H, Jia Y, Zhang Z, Craig R, Wang X, Elbein SC: Adiponectin receptor 1 gene (ADIPOR1) as a candidate for type 2 diabetes and insulin resistance. *Diabetes* 53:2132-2136, 2004

52. Staiger H, Kaltenbach S, Staiger K, Stefan N, Fritsche A, Guirguis A, Peterfi C, Weisser M, Machicao F, Stumvoll M, Haring HU: Expression of adiponectin receptor mRNA in human skeletal muscle cells is related to in vivo parameters of glucose and lipid metabolism. *Diabetes* 53:2195-2201, 2004

53. Civitarese AE, Jenkinson CP, Richardson D, Bajaj M, Cusi K, Kashyap S, Berria R, Belfort R, DeFronzo RA, Mandarino LJ, Ravussin E: Adiponectin receptors gene expression and insulin sensitivity in non-diabetic Mexican Americans with or without a family history of Type 2 diabetes. *Diabetologia* 47:816-820, 2004

54. Diez JJ, Iglesias P: The role of the novel adipocyte-derived hormone adiponectin in human disease. *Eur.J.Endocrinol.* 148:293-300, 2003

55. Brakenhielm E, Veitonmaki N, Cao R, Kihara S, Matsuzawa Y, Zhivotovsky B, Funahashi T, Cao Y: Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc.Natl.Acad.Sci.U.S.A* 101:2476-2481, 2004

56. Kondo H, Shimomura I, Matsukawa Y, Kumada M, Takahashi M, Matsuda M, Ouchi N, Kihara S, Kawamoto T, Sumitsuji S, Funahashi T, Matsuzawa Y: Association of adiponectin mutation with type 2 diabetes: a candidate gene for the insulin resistance syndrome. *Diabetes* 51:2325-2328, 2002

57. Menzaghi C, Ercolino T, Di Paola R, Berg AH, Warram JH, Scherer PE, Trischitta V, Doria A: A haplotype at the adiponectin locus is associated with obesity and other features of the insulin resistance syndrome. *Diabetes* 51:2306-2312, 2002

58. Lindsay RS, Funahashi T, Hanson RL, Matsuzawa Y, Tanaka S, Tataranni PA, Knowler WC, Krakoff J: Adiponectin and development of type 2 diabetes in the Pima Indian population. *Lancet* 360:57-58, 2002

59. Hotta K, Funahashi T, Bodkin NL, Ortmeyer HK, Arita Y, Hansen BC, Matsuzawa Y: Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. *Diabetes* 50:1126-1133, 2001

60. Hotta K, Gustafson TA, Yoshioka S, Ortmeyer HK, Bodkin NL, Hansen BC: Relationships of PPARgamma and PPARgamma2 mRNA levels to obesity, diabetes and hyperinsulinaemia in rhesus monkeys. *Int.J.Obes.Relat Metab Disord.* 22:1000-1010, 1998

61. Havel PJ: Update on adipocyte hormones: regulation of energy balance and carbohydrate/lipid metabolism. *Diabetes* 53 Suppl 1:S143-S151, 2004

62. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF: Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc.Natl.Acad.Sci.U.S.A* 98:2005-2010, 2001

63. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat.Med.* 7:941-946, 2001

64. Combs TP, Berg AH, Obici S, Scherer PE, Rossetti L: Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *J.Clin.Invest* 108:1875-1881, 2001

65. Ruan H, Lodish HF: Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha. *Cytokine Growth Factor Rev.* 14:447-455, 2003

66. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y: Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation* 102:1296-1301, 2000

67. Lihn AS, Pedersen SB, Richelsen B: Adiponectin: action, regulation and association to insulin sensitivity. *Obes.Rev.* 6:13-21, 2005

68. Qi Y, Takahashi N, Hileman SM, Patel HR, Berg AH, Pajvani UB, Scherer PE, Ahima RS: Adiponectin acts in the brain to decrease body weight. *Nat.Med.* 10:524-529, 2004

69. Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T, Matsuzawa Y: Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* 103:1057-1063, 2001

70. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE: The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat.Med.* 7:947-953, 2001

71. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T: Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat.Med.* 8:1288-1295, 2002

72. Gil-Campos M, Canete RR, Gil A: Adiponectin, the missing link in insulin resistance and obesity. *Clin.Nutr.* 23:963-974, 2004

73. Tomas E, Tsao TS, Saha AK, Murrey HE, Zhang CC, Itani SI, Lodish HF, Ruderman NB: Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proc.Natl.Acad.Sci.U.S.A* 99:16309-16313, 2002

74. Ceddia RB, Somwar R, Maida A, Fang X, Bikopoulos G, Sweeney G: Globular adiponectin increases GLUT4 translocation and glucose uptake but reduces glycogen synthesis in rat skeletal muscle cells. *Diabetologia* 48:132-139, 2005

75. Harman AW, McCamish LE, Henry CA: Isolation of hepatocytes from postnatal mice. *J Pharmacol.Methods* 17:157-163, 1987

76. Miyazaki T, Bub JD, Uzuki M, Iwamoto Y: Adiponectin activates c-Jun NH(2)-terminal kinase and inhibits signal transducer and activator of transcription 3. *Biochem.Biophys.Res.Commun.* 333:79-87, 2005

77. Shapiro L, Scherer PE: The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Curr.Biol.* 8:335-338, 1998