REGULATION OF ADIPONECTIN SECRETION BY ENDOTHELIN-1

AND MYOSIN II

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REGULATION OF ADIPONECTIN SECRETION BY ENDOTHELIN-1 AND MYOSIN II

DEEPA BEDI

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Deepa Bedi, daughter of Paramjeet Singh Bedi and Varjindra Pal Bedi, was born september, 1973, in Jabalpur, India. She graduated from Tashmi II state Medical College, Tashkent, Uzbekistan with the degree of Doctor of Medicine in Janaury, 1998. In the fall of 2001, she was accepted as a Cellular and Molecular Biology fellow at Auburn University. In 2003, she was awarded the Presidential Scholarship. On June 5, 2002, she married Manmeet Singh Chopra. Son of Harinder Singh Chopra and Kuldeep Chopra.

DISSERTATION ABSTRACT

REGULATION OF ADIPONECTIN SECRETION BY ENDOTHELIN-1

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Adiponectin is an adipokine with profound insulin-sensitizing, anti-inflammatory and anti-atherogenic properties. Plasma levels of adiponectin are reduced in insulin resistant states such as obesity, type 2 diabetes and cardiovascular diseases. Additionally, studies have shown that endothelin-1 (ET-1), a vasoconstrictor peptide, acutely stimulates and chronically inhibits adiponectin secretion. However, the mechanism by which ET-1 regulates adiponectin secretion is unknown. Therefore, we investigated the mechanism(s) responsible for the effects of ET-1 on adiponectin secretion. In order to determine the chronic effect of ET-1 on adiponectin secretion, 3T3-L1 adipocytes were treated for 24 hrs with ET-1 (10nM) and then stimulated with vehicle or insulin (100nM) for a period of 1-2 hrs. Chronic ET-1 (24 hrs) treatment significantly decreased basal and insulin

stimulated adiponectin secretion by 66% and 47%, respectively. Inhibition of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis by the PLCβ inhibitor, U73122, or exogenous addition of PIP_2: histone carrier (1.25μM: 0.625μM) ameliorated the decrease in basal and insulin-stimulated adiponectin secretion observed with ET-1. However, treatment with exogenous PIP_2: histone carrier complex and the actin depolymerizing agent latrunculin B (20μM) did not reverse the ET-1-mediated decrease in adiponectin secretion. In conclusion, we demonstrate that ET-1 inhibits basal and insulin-stimulated adiponectin secretion through PIP_2 modulation of the actin cytoskeleton. These studies provided evidence that actin cytoskeleton plays an important role in adiponectin secretion from adipocytes. In order to further elucidate the role of F-actin in the regulation of adiponectin secretion, we investigated the role of myosin II, an actin-based motor, in the trafficking and secretion of adiponectin in 3T3-L1 adipocytes. Myosin IIA and IIB isoforms were colocalized with adiponectin as determined by immunofluorescence and immunogold electron microscopy. Immunofluorescent and immunogold microscopy revealed that myosin IIA and IIB were dispersed throughout the cytoplasm of the cell while exhibiting perinuclear localization Inhibition of myosin II activity by blebbistatin or actin depolymerization by latrunculin B dispersed myosin IIA and IIB towards the periphery while significantly inhibiting adiponectin secretion. Therefore, the constitutive trafficking and secretion of adiponectin in 3T3-L1 adipocytes appears to occur by an actin-dependent mechanism that involves the actin-based motors, myosin IIA and IIB.

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CHAPTER 1. INTRODUCTION

Obesity is described as a state of increased body weight of sufficient magnitude to produce adverse health consequences (214). The increasing prevalence of obesity constitutes a major health problem in the United States, with more than two third of all adults currently overweight or obese. The percentage of overweight or obese adults in the United States increased from 45% in 1991 to 58% in 2001 (100). This increase in cases of obesity correlates with a significant increase in the cases of type 2 diabetes (106). According to American Diabetes Association, currently 20.8 million have been diagnosed with diabetes mellitus and approximately 90-95% of all diabetic patients are diagnosed with type 2 diabetes, or non-insulin dependent diabetes mellitus. More than 85% of all adult type 2 diabetic patients are classified as being either overweight or obese, with 55% of those patients are obese.

Traditionally, adipose tissue has been considered an energy storage organ, but it has now become apparent that adipose tissue is also an endocrine organ secreting a number of bioactive molecules known as adipocytokines or adipokines (79; 229). These adipokines mediate a wide variety of physiological functions in the body, including the maintenance of energy homeostasis, glucose and lipid metabolism and vascular homeostastis. In pathological conditions such as obesity, the secretion and expression of many of these adipokines are dysregulated resulting in imbalances in a wide variety of physiological functions.

An adipokine receiving widespread attention recently is adiponectin (or Acrp30). Adiponectin was first identified in 1995 by four independent laboratories. It is a 30kDa hormone produced exclusively by white adipose tissue. Adiponectin is one of the most abundant plasma proteins, circulating in the plasma in high concentrations ranging from 2-20 μg/ml and accounting for approximately 0.01-0.05% of total plasma proteins (8; 208). However, in obesity and type 2 diabetes, its expression and circulating levels are decreased (8; 88). Decreased adiponectin expression and secretion have been positively correlated with the decrease in insulin sensitivity in these disease states (89). Recently, adiponectin has been recognized to have not only insulin sensitizing properties but anti-atherogenic properties and anti-inflammatory as well. Additionally, adiponectin levels are decreased in patients with coronary heart disease and it has been suggested that it modulates the endothelial inflammatory diseases associated with coronary heart diseases (127). Therefore, adiponectin may provide a link between obesity, insulin resistance and cardiovascular disease and understanding the regulation of adiponectin secretion and gene expression is crucial to our knowledge of the metabolic syndrome.

Adiponectin expression and secretion from white adipose tissue is regulated by variety of factors. Factors such as TNF-α (167), interleukin-6 (184) and β-adrenergic agonists (246) decreases adiponectin secretion and factors such as insulin (16; 32; 208) and interleukin 15 (194) stimulate adiponectin secretion. However, the list of factors regulating adiponectin secretion is far from complete.

A recent study from our laboratory demonstrated that endothelin-1 (ET-1) has the ability to regulate adiponectin secretion. ET-1, a 21 amino acid peptide produced by endothelial cells of the vasculature, has mitogenic and vasoconstrictor properties (109).

Circulating levels of ET-1 are elevated in insulin resistant states such as type 2 diabetes, cardiovascular disease, renal disease (167), and in individuals with endothelial dysfunction (184). In all cases, a significant correlation between ET-1 levels and the severity of the disorder has been observed. Studies have demonstrated that elevated ET-1 may play a role in the development of insulin resistance in pathophysiologic conditions (246). ET-1 also affects adipose tissue protein secretion. ET-1 acutely stimulates leptin secretion from Ob-Luc cells and 3T3-L1 adipocytes (253) and alters resistin secretion from 3T3-L1 adipocytes (266). ET-1 has also been shown to acutely stimulate and chronically inhibit adiponectin secretion from 3T3-L1 adipocytes through the ET_A receptor (32). However, the mechanism(s) responsible for the observed effects has not been yet determined.

To investigate the mechanism by which ET-1 regulates adiponectin secretion and gene expression, 3T3-L1 adipocytes were acutely and chronically treated with ET-1. Adiponectin secretion into the media was determined by SDS-PAGE and immunoblotting, and adiponectin gene expression was analyzed by reverse-transcription followed by polymerase chain reaction (RT-PCR). Chronic treatment with ET-1 inhibited basal and insulin-stimulated adiponectin secretion. Gene expression analysis of adiponectin revealed that the chronic inhibitory effects of ET-1 on adiponectin secretion are not mediated by concomitant changes in gene expression. Since, ET-1 has been previously shown to affect vesicular trafficking of proteins by modulation of the actin cytoskeleton in 3T3-L1 adipocytes, we investigated whether ET-1's modulation of the actin cytoskeleton affects intracellular trafficking and secretion of adiponectin. The results of these studies suggest that elevated levels of ET-1 in the obese and diabetic state

may contribute to decreases in circulating adiponectin levels and decreased insulin sensitivity through impairment of the actin cytoskeleton. In addition to revealing the role of ET-1 in adiponectin secretion, these studies also demonstrated the importance of the actin cytoskeleton in the constitutive secretion of adiponectin. Actin cytoskeleton has been implicated in the vesicular trafficking at the level of Golgi via actin binding proteins (82). These actin-binding proteins binds to the Golgi-derived vesicles and faciliates their translocation and tethering to the cortical F-actin. One such protein is myosin II that is linked to Golgi membrane and is involved in the constitutive transport of proteins (165). We therefore looked at the role of myosin II in the intracellular trafficking and secretion of adiponectin. The results of these studies more clearly define the specific events associated with the vesicular trafficking of adiponectin.

CHAPTER II. REVIEW OF LITERATURE

Metabolic Syndrome

The concurrence of disturbed glucose metabolism, increased abdominal fat distribution, dyslipidemia and hypertension and its association with subsequent development of type 2 diabetes mellitus and cardiovascular disease has given rise to the concept of the metabolic syndrome (195). The World Health Organization (WHO) definition of the metabolic syndrome includes waist-hip ratio > 0.90 or body mass index >30 kg/m^2 and is the most sensitive of the definitions for diabetes (128). Insulin resistance is considered to be the primary cause of the cluster of these disorders. The metabolic syndrome was defined as insulin resistance in the top 25 percent of the population as measured by the euglycemic-hyperinsulinemic clamp or the presence of impaired glucose tolerance or type 2 diabetes and the presence of at least two of the following: abdominal obesity (waist-hip ratio > 0.90 or body mass index >30 kg/m^2), dyslipidemia (serum triglycerides >1.70 mmol/liter or high density lipoprotein (HDL) cholesterol < 0.9 mmol/liter), hypertension (>160/90 mmHg), or microalbuminuria. These core components were considered most suitable for a general definition, although many other disturbances, including disorders of coagulation and endothelial function, hyperuricemia, and elevated leptin level—have been associated with the metabolic syndrome (139). Figure 1 shows the proposed model of the metabolic syndrome and its component.

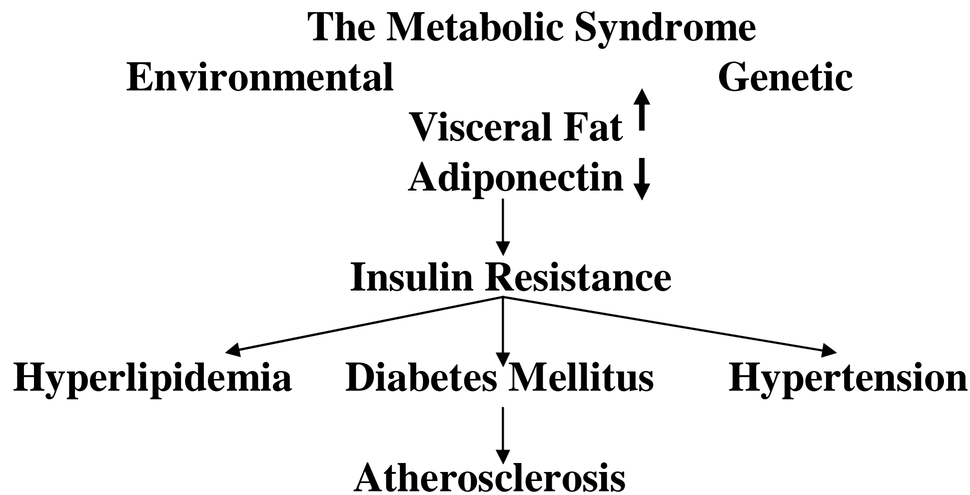

Figure 1. Concept of Metabolic syndrome. From Matsuzawa et al., *Arterioscler. Thromb. Vasc. Biol.* 2004; 24; 29-33.

Insulin resistance

A major component of the metabolic syndrome is insulin resistance. Insulin resistance is defined as resistance to the physiological actions of insulin at its target tissue, primarily skeletal muscle, liver and adipose tissue. Insulin resistance decreases the ability of insulin to promote glucose metabolism and hepatic glucose clearance (107). Insulin resistance is a hallmark characteristic of both type 2 diabetes and obesity. Insulin resistance causes elevations in blood glucose levels that are compensated, at least partially, by increased insulin secretion. This leads to impaired glucose tolerance and hyperinsulinemia. Eventually, beta cells lose their capacity to secrete insulin and hyperglycemia occurs which can manifest type 2 diabetes (17; 174). Insulin resistance

combined with β-cell failure leads to the decompensated hyperglycemic diabetic state (107).

Type 2 Diabetes Mellitus

The metabolic syndrome frequently leads to development of type 2 diabetes mellitus. Diabetes is a chronic metabolic disorder affecting approximately 5% of the population in the industrialized nations (159). Diabetes occurs in all populations and affects all age groups. It is the sixth leading cause of death in the United States (48). Decrease in insulin secretion due to autoimmune destruction of beta cell is responsible for type 1 diabetes mellitus. Type 1 diabetes is normally diagnosed in young children or adolescents, however it has also been diagnosed in adults. The more prevalent form, type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM), accounts for more than 90% of cases. The pathogenesis of type 2 diabetes is complex, involving progressive development of insulin resistance and a relative deficiency in insulin secretion, leading to overt hyperglycemia (225). Type 2 diabetes is most commonly diagnosed in adults, however it is becoming more frequently diagnosed among children and adolescents. Type 2 diabetes has become one of the major causes of premature illness and death , mainly through the increased risk of cardiovascular disease (CVD) which is responsible for 80% of these deaths (100).

Obesity

Obesity is defined as an excess of body fat, but is clinically difficult to define. One generally accepted measure of obesity is body mass index or BMI. BMI is calculated as a person's body weight in kilograms divided by their height in meters squared (kg/m^2).

Overweight is defined by a BMI ≥ 25kg/m^2 while *obesity* is defined by a BMI ≥ 30 kg/m^2 (1). However, the sole use of BMI as an indicator of obesity does not take into account body fat distribution or difference in body composition, which is considered to be an independent predictor of health risk (9; 160).

Obesity has reached epidemic proportions in the United States and the incidence of obesity continues to increase in the U.S. and around the world. Statistics from a study conducted by National Health Nutronal Examination Survey confirms that the prevalence of obesity among adults overall in the United States increased from 22.9% during 1988--1994 to 30.5% during 1999--2002; the prevalence of obesity among adults with diagnosed diabetes remained high, at 45.7% during 1988--1994 and 54.8% during 1999—2002 (61; 173). The type 2 diabetes epidemic is believed to be largely due to a concomitant increase in obesity levels, because population increases in diabetes have coincided with increases in obesity and because weight gain is a key determinant of insulin resistance and diabetes (159). Alarmingly, one in six children and adolescents is overweight (48). Obesity and diabetes are recognized as major causes of morbidity and mortality in the United States.

Obesity, especially visceral obesity, is considered a major risk factor for the development of metabolic abnormalities such as dyslipidemia, glucose intolerance and finally type 2 diabetes (73). In addition, obesity is the major underlying risk factor for CVD. It is associated with multiple risk factors, and it is also a risk factor for type 2 diabetes. However, the molecular link between obesity and the development of disease is far from understood.

Adipose tissue

Adipose tissue is specialized connective tissue that functions as the major storage site for fat in the form of triglycerides. Adipose tissue is found in mammals in two different forms: white adipose tissue (WAT) and brown adipose tissue (BAT). The concept of adipose tissue as a passive organ of energy storage to an active participant in hormonal regulation of homeostatic systems has occurred relatively recently (158).

Adipose tissue metabolism

Adipose tissue metabolism is influenced by several factors. Hormones such as insulin and non-humoral agents such as norepinephrine regulate adipose tissue metabolism (235). Fatty acids are the major secretory products of WAT. Fatty acids are synthesized *de novo* in adipocytes or obtained from plasma triglycerides (235). In mature adipocytes, insulin promotes triglyceride storage by stimulating glucose uptake and conversion of acetyl-CoA into triglycerides as well as inhibiting lipolysis. White fat also stores cholesterol and is involved in the metabolism of steroid hormones.

Adipose tissue is no longer viewed as a passive repository for triacylglycerol storage and a source of free fatty acids (FFAs). As developing preadipocytes differentiate to become mature adipocytes, they acquire the ability to synthesize proteins, many of which are released as enzymes, cytokines, growth factors and hormones involved in a variety of physiological processes. Adipose tissue plays an important role in maintaining whole body energy homeostastis. Mature adipocytes are widely acknowledged as an active endocrine and paracrine organ, secreting an ever-increasing number of mediators that participate in diverse metabolic processes (59; 130; 158).

The importance of adipose tissue as an endocrine organ and its role in the maintenance of energy homeostasis has become more and more apparent with increasing incidences of obesity, or excess adipose tissue, as well as in cases of lipodystrophies, or insufficient adipose tissue. Excess body fat not only leads to changes in fat tissue development and growth and the induction of insulin resistance, but also leads to endothelial dysfunction via the proinflammatory and prothrombotic effects of adipokines, which can lead to metabolic syndrome.

Adipose-derived proteins

Adipocytes produce a great number of factors known as adipose-derived proteins or adipokines. Circulating adipokines such as adiponectin, resistin and leptin are hormones released only from mature adipocytes, while other adipokines such as IL-1, IL-8, TNF-α, PAI-1 and IL-6 are released from both adipocytes as well as non-fat cells (49). White adipose tissue lies at the heart of a network of autocrine, paracrine, and endocrine signals which interacts with neuroendocrine regulators that enable the body to adapt to a range of different metabolic challenges, such as starvation, stress, infection, gross energy excess and many others.

Although adipose tissue was identified as a major site of metabolism for sex steroids as early as 1987, it was not until the identification of leptin in 1994 that solidified the existence and importance of adipose-derived endocrine factors. It was studies that leptin informs the brain about the abundance of body fat, thereby allowing feeding behaviour, metabolism and endocrine physiology to be coupled to the nutritional status of the organisms that demonstrated the important role of adipokines (3; 65).

It is now recognized that adipokines have many different functions in the body. Table 1 describes many of the known adipokines and their endocrine functions. These functions include sending signals regarding the nutritional status of the animal (leptin, adiponutrin), maintaining insulin-senstivity (adiponectin) immune-related functions (interlukin-6), and regulation of blood flow (angiotensinogen).

Table 1. Adipocyte-derived proteins and their functions.

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

Modified from Kershaw and Flier, *The Journal of Endocrinolgy and Metabolism* 2004. 89(6):2548-2556.

Adiponectin

Discovery

Adiponectin is a 30 kDa protein secreted abundantly by adipocytes. Adiponectin was originally identified independently by four different laboratories using different approaches. In 1995, Scherer *et al*. identified a protein using subtractive cDNA screening to identify mRNAs induced during differentiation of 3T3-L1 adipocytes. Northern blot analysis of 250bp clone showed a 100-fold increase in induction during differentiation; the resulting full-length cDNA was isolated and sequenced. The encoded protein, Acrp30, was novel; it contained 247 amino acids with a predicted molecular mass of 28 kDa. Acrp30 consists of a predicted amino-terminal signal sequence, followed by a stretch of 27 amino acids. The carboxyl-terminal globular domain exhibits striking homology to a number of proteins, such as the globular domains of type VIII and type X collagens, the subunits of complement factor C1q and a protein found in the serum of hibernating animals during the summer months (208). Scherer *et al*. were the first to suggest that Acrp30 secretion is acutely regulated by insulin, as well as the fact that it is a very abundant plasma protein, accounting for approximately 0.05% of total plasma proteins (208).

In 1996, Hu *et al.* used mRNA differential display to isolate a novel adipose cDNA they named adipoQ (91). The encoded protein consisted of 247 amino acids and was specific to adipose tissue in both mouse and rat. AdipoQ was found to be highly regulated during the differentiation of adipocytes, and Hu *et al*. were the first to describe a significant down-regulation of adipoQ mRNA in fat tissues collected from obese

humans and mice (91). In 1996, Nakano *et al*. used gel chromatography to identify a novel protein from human plasma which was named gelatin binding protein of 28kDa or GBP28 (166). GBP28 was identified by its affinity to gelatin-Cellulofine. The protein purified from the column was described as having a molecular mass of 28kDa under reducing conditions, and 68kDa under non-reducing conditions (166). The amino acid sequence for GBP28 showed 82.7% homology to Acrp30, which is thought to be the murine equivalent of GBP28 (166).

Maeda *et al*. isolated a novel adipose-specific gene, Adipose Most Abundant Gene Transcript (apM1), from human adipose tissue (144). Northern blotting of RNAs from several different human tissues including skeletal muscle, small intestine, placenta, uterus, ovary, kidney, liver, lung, brain, heart and bladder revealed that apM1 was specifically expressed in adipose tissue and encodes the adiponectin protein.

Protein structure

Human adiponectin consists of 244 amino acid residues (208) and is comprised of an N-terminal signal sequence, a non-homologous region, an amino-terminal collagenous domain and a domain containing 22 Gly-X-Pro or GLY-X-X repeats. This suggests that adiponectin has a straight collagen stalk as opposed to the kinked collagen domain present in the protein C1q (208). There is a globular domain in the carboxy-terminus of the protein that bears structural homology to a number of other proteins including the globular domains of type VIII and type X collagens, and the subunits of complement factor C1q, and TNF-α (Figure 2) (144; 208). The three-dimensional structure of adiponectin is very similar to that of TNF-α (91).

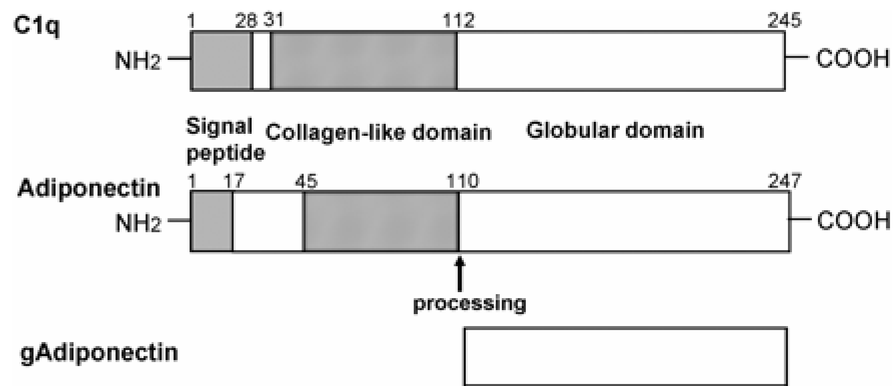

Figure 2. Structure and domain of adiponectin. From Kadowaki et al., From Endocrine reviews 26(3); 439-451.

Adiponectin is secreted from adipocytes and post-translationally modified into many different isoforms (241). Two-dimensional gel electrophoresis analysis identified eight protein spots that were preferentially expressed and secreted by adipocytes, and not from undifferentiated 3T3-L1 preadipocytes Two-dimensional gel electrophoresis of recombinant adiponectin produced by *E. coli* revealed only one isoform of adiponectin suggesting that multiple isoforms of adiponectin are the result of post-translational processing in mammalian adipocytes (241). Carbohydrate-based detection of proteins separated by two-dimensional gel electrophoresis revealed that six isoforms of adiponectin derived from adipocytes are glycosylated, suggesting that glycosylation may at least partly contribute to the heterogeneity of adiponectin. The four conserved lysines (residues 68, 71, 80, and 104) in the collagenous domain were identified as potential glycosylated sites. Although there are two consensus N-linked glycosylation sites (Asn^{53}

and Asn^{233}), treatment with tunicamycin, an inhibitor of N-linked glycosylation, did not affect the glycosylation pattern, thus excluding the possibility of N-linked glycosylation of adiponectin (241). Amino acid analysis revealed that all four lysines were hydroxylated. Replacement of the lysine residues with arginine residues resulted in decreased action of insulin to suppress hepatic glucose production indicating that the glycosylated lysine residues are required for the biological activity of adiponectin (240). In later studies, Wang *et al*. demonstrates that the four lysines (residues 68, 71, 80, and 104) in the collagenous domain of adiponectin are critical for its insulin-sensitizing activity with respect to inhibition of hepatic glucose production. These four lysine residues were found to be hydroxylated and glycosylated, thus emphasizing the important role of post-translational modifications in the biological activities of adiponectin (240).

Adiponectin circulates in the plasma in structures called protein complexes or homomultimers. Monomers are single 30kDa proteins which can form trimers through associations in their globular domains (13). Trimers associate with other trimers through interactions in their collagen domains to form higher molecular weight structures (Figure 3) (181). Adiponectin in human or mouse serum and adiponectin expressed in NIH-3T3 cells forms a wide range of multimers from trimers (90 kDa) and hexamers (180 kDa) to higher molecular weight (HMW) multimers (> 400kDa) such as dodecamers and 18 mers (181). Adiponectin can exist as full length or smaller, globular fragment; however almost all adiponectin has been identified in human plasma at physiologically appears to exist as full length in plasma. It has been proposed that globular fragment is generated by proteolytic cleavage by leukocytes elastase secreted from activated monocytes (239). Several previous studies have demonstrated the necessity of disulfide bonds mediated by

Cys^{39} in the formation of hexameric adiponectin and HMW adiponectin (181). A recent study by Wang *et al.,* has provided more evidence to the oligomers formation of adiponectin both *in vivo* and *in vitro* to support the notion that post-translational modifications, specifically the hydroxylation and further glycosylation of several lysine residues within the collagenous domain, are required for intracellular assembly of the HMW adiponectin oligomers. For example, ablation of hydroxylation and glycosylation by substitution of the four lysines (residues 68, 71, 80, and 104) with arginines impeded the intracellular assembly of the HMW adiponectin in both the cell culture system and mice (240).

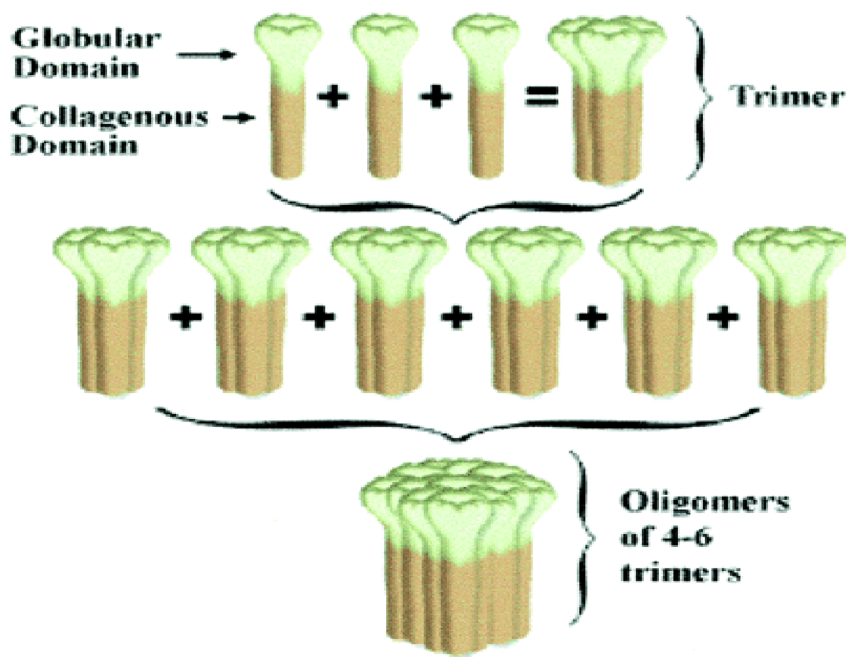

Figure 3. Model for assembly of adiponectin complexes. Adapted from Chandran et al. *Diabetes Care.* 2003.26(8):2442-2450.

Adiponectin receptors

Yamauchi *et al*. were the first to describe functional receptors for adiponectin in 2003. They isolated cDNA for adiponectin receptors (AdipoR) that mediate the antidiabetic effects from human skeletal muscle by screening for globular adiponectin binding and named them AdipoR1 and AdipoR2 (256). Although these receptors are expressed ubiquitously, AdipoR1 is more highly expressed in skeletal muscle and AdipoR2 is more highly expressed in the liver. Scatchard plot analysis revealed that AdipoR1 is a high-affinity receptor for globular adiponectin and a low-affinity receptor for full-length adiponectin while AdipoR2 is an intermediate receptor for both globular and full-length adiponectin (256). The full-length form of the protein is believed to be the circulating form of adiponectin. The globular domain of the protein has been demonstrated to have more potent effects on fatty acid oxidation in skeletal muscle than the full-length protein (257).

AdipoR1 and AdipoR2 appers to be integral membrane proteins. The N terminus of the adiponectin receptor lies on the internal surface of the cell membrane, while the C terminus lies on the external surface, which is distinct from G-protein coupled receptors (256). Figure 4 shows the proposed structure of adiponectin receptors.

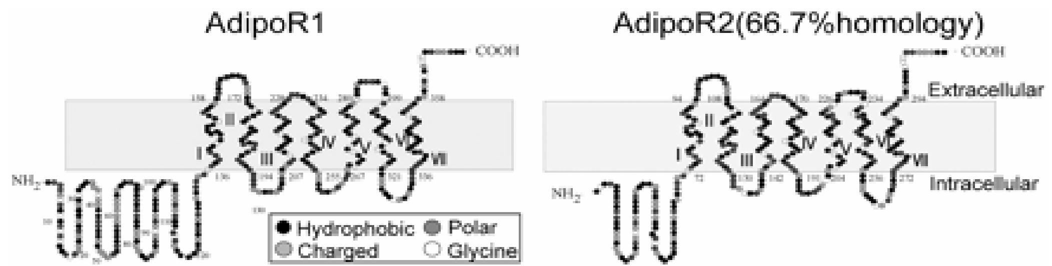

Figure 4. Proposed structure of adiponectin receptors. From Kadowaki et al., From Endocrine reviews 26(3); 439-451.

It has been reported that binding of globular and full-length adiponectin to AdipoR1 or AdipoR2 increases PPAR-α ligand activity, and increases the phosphorylation of adenosine monophosphate protein kinase (AMPK), acetyl coenzyme A carboxylase (ACC), and p38 mitogen-activated protein kinase. These events result in increased fatty acid oxidation and increased glucose uptake in skeletal muscle (AdipoR1) and decreased glucose output by the liver (AdipoR2) (256).

Recent *in vivo* and *in vitro* studies have investigated whether AdipoR1 and AdipoR2 expression levels are altered in physiological/ or pathophysiological states. The levels of AdipoR1 and AdipoR2 mRNA expression in both liver and skeletal muscle increased after fasting, and refeeding restores these levels. The levels of AdipoR1 and AdipoR2 mRNA were increased in mice subjected to streptozotocin-induced diabetes due to hypoinsulinemia/hyperglycemia and were completely restored by insulin treatment (231). The expression of both AdipoR1 and AdipoR2 were decreased in muscle and adipose tissue, but not in liver of insulin resistant ob/ob mice, which exhibited hyperglycemia and hyperinsulinemia, as compaed to control mice (231). They also

suggest that downregulation of adiponectin receptors in *ob/ob* mice is correlated with decreased adiponectin sensitivity.

Civitarese *et al.* suggested that AdipoR1and AdipoR2 expression levels in muscle are lower in subjects with a family history of type 2 diabetes than those without, and that the expression levels of both receptors correlated positively with insulin sensitivity (31). While studies done by Debard *et al*. did not demonstrate any significant differences in gene expression levels of AdipoR1 or AdipoR2 in skeletal muscle of type 2 diabetic patients (41). Studies done by Staiger *et al*. showed the expression levels of AdipoR1 and AdipoR2 from human myotubes and that neither AdipoR1 nor AdipoR2 correlated with insulin sensitivity (215). Ouchi *et al*. showed that obesity decreases the expression levels of AdipoR1/R2, thereby reducing adiponectin sensitivity, which leads to insulin resistance and the so called "vicious cycle" (179). McAinch *et al.* reported that adiponectin and leptin are rapid and potent stimulators of AdipoR1 in myotubes derived from lean healthy individuals. This effect was abolished in myotubes derived from obese, obese diabetic subjects, and obese-prone individuals who had lost significant weight after bariatric surgery (156). The inability of skeletal muscle of obese and diabetic individuals to respond to exogenous adiponectin and leptin may be further suppressed as a result of impaired regulation of the AdipoR1 gene (156). Adiponectin receptors have been reported to be upregulated in endstage renal diseases (209), polycystic ovarian syndrome (223). Further investigation of the expression of AdipoR1 and AdipoR2 will be necessary to elucidate whether there is indeed a correlation between adiponectin receptor levels and insulin sensitivity. In 2004, the existence of another set of adiponectin receptors called the T-cadherins was reported by Hug *et al*. (93). They are

members of the cadherin family of receptors involved in calcium-mediated cell-cell interactions, and are structurally distinct from AdipoR1 and AdipoR2. T-cadherin receptors are expressed on smooth muscle cells, as well as endothelial cells, and bind eukaryotically-produced high molecular weight, as well as hexameric forms of adiponectin, but not the trimeric or globular forms of adiponectin. They do not bind to bacterially produced adiponectin, suggesting that post-translational modifications are necessary for the binding of adiponectin to the T-cadherin receptors (93).

Physiological functions of adiponectin

A wide array of physiological functions in a variety of tissues including skeletal muscle, liver and the vasculature occur following adiponectin binding to its receptors. Adiponectin possesses insulin-sensitizing effect and can be an important link between obesity, insulin resistance and the development of type 2 diabetes. Adiponectin levels are reduced in obese adults as well as adults with type 2 diabetes (88; 154). More recent studies suggest that low adiponectin levels are more strongly correlated with insulin resistance and hyperinsulinemia than degree of adiposity and glucose tolerance (216; 244). Low basal levels of adiponectin in a population of Pima Indians were found to predict decreased insulin sensitivity in this population regardless of adiposity (142).

There is an abundant amount of experimental evidence supporting the role of adiponectin as an insulin-sensitizing hormone. Adiponectin was able to improve insulin resistance in KKA^y mice (KK mice overexpressing the agouti protein), as a model of the metabolic syndrome and type 2 diabetes linked to obesity. Plasma adiponectin levels were decreased in KKA^y mice fed a high-fat diet. Replenishment of adiponectin

significantly ameliorated high-fat diet–induced insulin resistance and hypertriglyceridemia, which led to propose that adiponectin is an insulin-sensitizing adipokine (258). Scherer and colleagues reported that an acute increase in the level of circulating adiponectin leads to a transient decrease in basal glucose level by inhibiting both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production in both wild-type and type 2 diabetic mice, and they proposed that adiponectin sensitizes the body to insulin (12). Lodish and colleagues reported that a proteolytic cleavage product of adiponectin, which structurally resembles globular adiponectin, increases fatty-acid oxidation in muscle, decreases plasma glucose, and causes weight loss in mice (63). Intraperitoneal (ip) injection of adiponectin lowered serum glucose levels and free fatty acids without changes in insulin levels in mice (12). Chronic injections of adiponectin resulted in decreased body weight in high-fat-fed mice and improved insulin sensitivity in insulin resistant mice (260). The adiponectin-deficient mice examined by Maeda *et al.* exhibited a near-normal insulin sensitivity when fed a standard laboratory diet, but developed severe insulin resistance, especially in skeletal muscle on a high-fat, high-sucrose diet (145). Ma *et al.* reported that adiponectin-deficient mice displayed increased fatty-acid oxidation in skeletal muscle, but showed no effect on either insulin sensitivity or glucose tolerance whether on a standard or a high-fat diet (143). These studies suggests that the insulin resistance associated with high fat diets and obesity is caused at least in part by the decrease in adiponectin and that reduction in adiponectin plays casual roles in the development of insulin resistance.

Skeletal muscle is a major target for adiponectin's physiological functions. Adiponectin reduces tissue triglyceride content and up-regulates insulin signaling. In skeletal muscle, adiponectin increases expression of molecules involved in fatty-acid transport such as CD36, in combustion of fatty-acid such as acyl-coenzyme A oxidase, and in energy dissipation such as uncoupling protein 2. These changes lead to decreased tissue TG content in skeletal muscle (258).

Yamauchi *et al*. demonstrated that globular and full-length adiponectin phosphorylated and activated AMPK in skeletal muscle. Activation of AMPK resulted in phosphorylation of acetyl coenzyme A carboxylase, (ACC), fatty acid oxidation, glucose uptake and lactate production in C2C12 myocytes. A recent study by Li *et al.* showed that adiponectin modulates carnitine palmitoyltransferase-1 through AMPK signaling cascade in rat cardiomyocytes (138). Adiponectin increases the expression of PPARα, which in part is responsible for adiponectin action on increased fatty acid oxidation and energy dissipation, which leads to decreased TG content in the skeletal muscle and thus increases insulin sensitivity *in vivo* (258).

Liver is another primary target organ of adiponectin's insulin sensitizing properties. Adiponectin enhanced insulin action in primary hepatocytes by decreasing gluconeogenesis and hepatic glucose output (12). However, exposure to the isolated globular C-terminus did not have the same metabolic effects in isolated hepatocytes, indicating that the full-length protein is necessary for adiponectin's insulin-sensitizing effects in the liver (12). Adiponectin mediates these insulin-sensitizing effects by increasing fatty acid oxidation through activation of AMPK and PPAR-α (257). Activation of AMPK in the liver is followed by increased phosphorylation of ACC,

decreased malonyl-CoA and increased fatty acid oxidation (257). Activation of PPAR- α also results in increased gene expression of enzymes involved in fatty acid oxidation. Scherer *et al.* reported that in adiponectin transgenic mice (36), reduced expression of gluconeogenic enzymes such as phosphoenolpyruvate carboxylase and glucose-6-phosphatase is associated with elevated phosphorylation of hepatic AMPK, which may account for inhibition of endogenous glucose production by adiponectin (33).

Adiponectin has been reported to have anti-inflammatory and antiatherosclerotic effects. Adiponectin was demonstrated to strongly inhibit the expression of adhesion molecules, including intracellular adhesion moleculae-1, vascular cellular adhesion molecule-1, and E-selectin. Adiponectin was also shown to inhibit TNF-α-induced nuclear factor-κB activation through IκB phosphorylation (179). Suppression of nuclear factor- κB by adiponectin might play a major molecular mechanism for the inhibition of monocyte adhesion to endothelial cells (177). Adiponectin also inhibits the expression of the scavenger receptor class A-1 of macrophages, resulting in decreased uptake of oxidized low-density lipoprotein by macrophages and inhibiton of foam cell formation (178). Recently, selective suppression of endothelial cell apoptosis via AMPK activation by the HMW form of adiponectin has been reported (121). Interestingly, diabetic patients with coronary artery disease (CAD) had lower levels of adiponectin than diabetic patients without CAD (26; 88) suggesting that adiponectin deficiency may play a role in the development of CAD.

Qi *et al.* recently demonstrated that adiponectin is present and has effects in the brain (191). Their results suggest that adiponectin is transported from the serum to the cerebral spinal fluid (CSF). Intracerebroventricular (ICV) injections of adiponectin in

normal mice reduced the body weight of these mice without affecting food intake. Increased oxygen consumption as well as increased expression of uncoupling protein-1 (UCP-1) in brown adipose tissue was also observed in these mice. These results suggest that ICV injections of adiponectin increased thermogenesis in these animals. Importantly, ICV injections of adiponectin in Agouti mice did not have any effects on body weight or thermogenesis. This suggests that these effects of adiponectin are mediated at least in part through the melanocortin pathway (191). Figure 3 represents a hypothetical model for the actions of adiponectin in its target tissues including liver, skeletal muscle, brain and the endothelium (10).

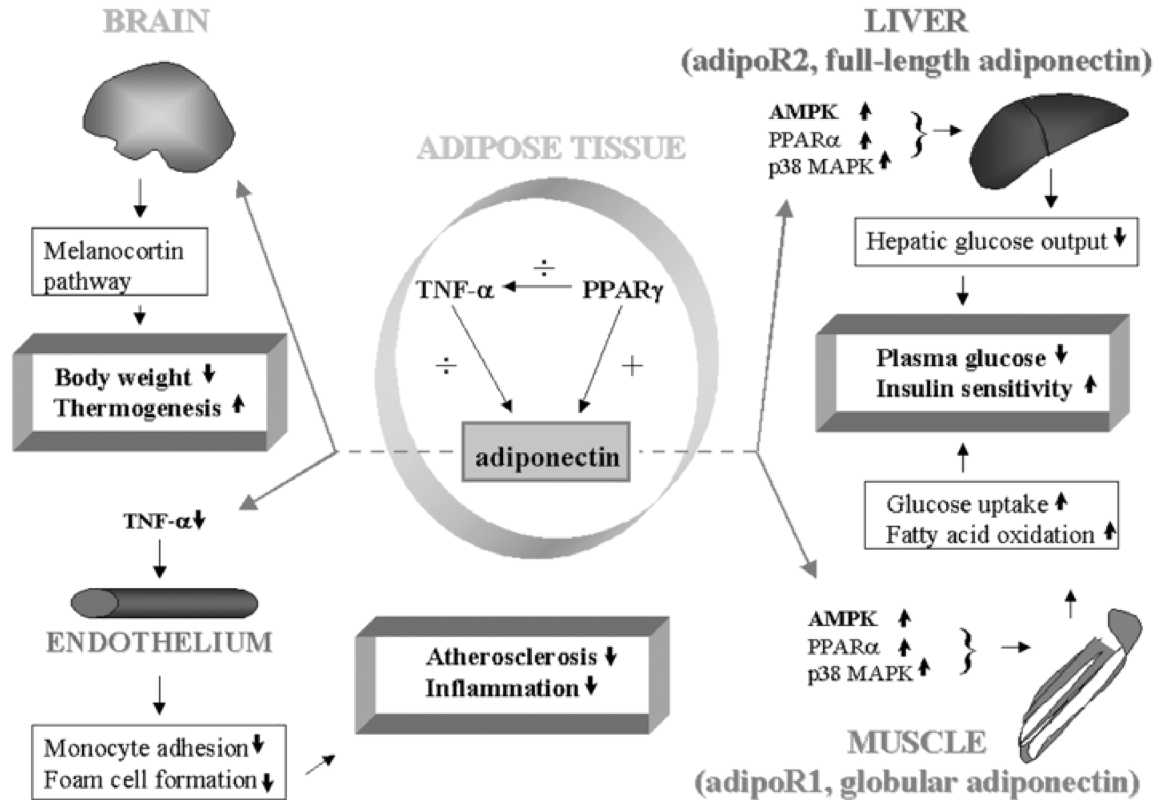

Figure 5. Illustration of target tissues and proposed mode of action and regulation of adiponectin. From Lihn A. S. *et al. Obesity Reviews.* 2005. 6, 13-21.

Role of HMW adiponectin in insulin resistance and type 2 diabetes.

Several observations support the hypothesis that HMW adiponectin is the more active form of the protein and has a more relevant role in insulin sensitivity and in protecting against diabetes. Rare mutations — G84R and G90S — in the collagen domain are closely associated with type 2 diabetes (181; 224). Subjects with either of these 2 mutations have extremely low levels of HMW adiponectin. Increases in the ratio of plasma HMW adiponectin levels to total adiponectin levels correlate with

improvement in insulin sensitivity during treatment with an insulin-sensitizing drug, TZD, in both mice and human diabetic patients, whereas increases in total serum adiponectin levels do not show good correlations with improvement in insulin sensitivity during treatment with TZD at the individual level (182). The ratio of plasma HMW adiponectin to total adiponectin correlated more significantly with glucose and insulin levels than did the total adiponectin level (129). Levels of total adiponectin, HMW adiponectin, LMW adiponectin, and the HMW-to-total adiponectin ratio all correlated significantly with key features of central obesity and the insulin-stimulated glucose disposal rate (60). These studies suggest that measurement of the HMW adiponectin level may be superior to measurement of total adiponectin.

Regulation of adiponectin

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

Adiponectin levels are influenced by many factors. Several studies have indicated that insulin acutely stimulates adiponectin secretion (16; 32; 208). Longer term incubation (24 hrs) with insulin, however, has been shown to increase or decrease both adiponectin secretion and gene expression (207). Interlukin -6 and TNF-α are cytokines

produced from adipose tissue, and levels of these cytokines are increased in insulin resistance and obesity. Both cytokines inhibit adiponectin gene expression and secretion from adipocytes (53; 54). Adiponectin gene expression is also inhibited by ß-adrenergic agonists (43; 52), and glucocorticoids (51; 77). Adrenalectomy increases adiponectin gene expression and circulating adiponectin levels, along with insulin sensitivity in *ob*/*ob* mice (147). The effects of cytokines, catecholamines, and glucocorticoids to induce insulin resistance could be mediated, in part, by their effects to decrease adiponectin production.

Androgens decrease plasma adiponectin and androgen-induced hypoadiponectinemia may be related to the high risks of insulin resistance and atherosclerosis in men (172). Xu *et al.* showed that testosterone selectively reduces the HMW form of adiponectin by inhibiting its secretion from adipocytes (254).

Other hormonal regulatory factors of adiponectin include insulin-like growth factor (IGF-1), growth hormone, and leptin. Growth hormone increases adiponectin gene expression and secretion (53). Leptin, another adipokine, has been shown to increase adiponectin expression and secretion in ob/ob mice (42). However, another study showed that central leptin gene therapy administered in *ob/ob* and wild type mice decreased plasma levels of adiponectin without an alteration in TNF-α levels (69). In addition, peripheral administration of leptin also significantly reduced plasma levels of adiponectin in *ob/ob* and wild type mice (69). Studies done by Delporte *et al*. showed that beta-agonists inhibited adiponectin production and maturation, and thus exerted a dual (pre- and post-translational) negative effect on adiponectin secretion by cultured mouse adipose explants (43). Pharmacological agents have also been shown to affect

adiponectin gene expression and protein secretion. PPARγ ligands such as the anti-diabetic class of drugs thiazolidinediones (TZDs) are known to enhance insulin sensitivity in insulin resistant type 2 diabetic animal models as well as insulin resistant patients. Recent studies have shown that TZDs increase adiponectin expression and secretion from adipocytes (262). Therefore, it is believed that at least a portion of the insulin-sensitizing properties of the TZDs results from increased circulating levels of adiponectin.

Table 2. Factors influencing adiponectin secretion and gene expression.

FACTOR	**ADIPONECTIN GENE EXPRESSION**	**ADIPONECTIN SECRETION**
Insulin	+/-	+/-
PPARγ ligands/TZDs	+	+
TNF-α	-	-
Glucocorticoids	-	Not determined
β-adrenergic agonists	-	Not determined
cAMP	-	-
Interlukin-6	-	-
Growth Hormone	+	+
Exercise	Not determined	+
Leptin	+	-
Free Fatty Acids	Not determined	-
Weight loss	+	+

Modified from Stefan and Stumvoll. *Hormone and Metabolic Research.* 2002.34:469-474.

There appear to be gender-related differences in the circulating concentrations of adiponectin. Adult female mice have higher Acrp30 levels than males, in agreement with the sexual dimorphism in humans (8; 88; 262). Plasma adiponectin levels are 35% lower in human males compared to human females (6.0 μg/ml vs. 9.1μg/ml) (172). There are no significant differences in plasma adiponectin levels between pre- and post-menopausal women (172). This suggests that male sex hormones are a potential regulator of circulating adiponectin levels. Mature ovaries have a mild, but significant influence on adult Acrp30 levels. OVX in young cycling mice induced plasma Acrp30 (10 days after surgery), and E2 implants reversed the effect (35).

Adiponectin has a relatively long half-life in serum (2.5-6 h) (87; 181), and it was recently determined that adiponectin levels are also determined in part by ultradian patterns. In other words, adiponectin is secreted with diurnal variations in less than a 24 hour period (69). In normal human males, serum adiponectin was characterized by a nocturnal decline starting in the late evening and reaching its maximum decrease in the early morning (~ 3am; 3.56 ± 0.3 μg/ml) (69). Serum adiponectin levels were higher during the day with peak levels reached at approximately 11am (5.28 ± 0.3 μg/ml) (69).

The effects of weight loss and exercise have also been investigated as potential mediators of adiponectin secretion and expression. Circulating adiponectin levels increases after weight loss in humans (88). The low plasma adiponectin concentrations in morbidly obese subjects are normalized after weight loss induced by gastric bypass surgery (50; 263). In another study, obese men (BMI >35 kg/m^2) were placed on a calorie restricted diet for 20 weeks. Prior to weight loss, plasma adiponectin levels were 47% lower in obese mean compared to lean individuals (24); following diet-induced

weight loss of approximately 20kg, plasma adiponectin levels increased by 51% (2.3 ± 0.6 vs. 3.4 ± 0.8 mg/l). Adiponectin mRNA was collected from adipose tissue biopsies from these patients before and after weight loss, and following weight reduction, adiponectin mRNA levels increased by 45%. In both of these studies, the increases in plasma adiponectin levels were significantly correlated with increases in insulin sensitivity (263).

Other studies provide further evidence for the dysregulation of adiponectin in obesity and diabetes. Hu *et al*. demonstrated that apM1 gene expression is dysregulated in obesity. A reduction in apM1 mRNA was observed in both obese humans and mice as compared to normal controls (91). Arita *et al*. measured plasma adiponectin from non-obese and obese subjects and found that circulating levels of this protein are significantly reduced in obese subjects (8.9µg/ml vs. 3.7µg/ml) (8). Thiazolidiones (TZDs) such as rosiglitazone and pioglitazone, are a class of anti-diabetic drugs which have been shown to improve insulin sensitivity. Administration of TZDs to obese mice and cultured 3T3-L1 adipocytes increased mRNA expression of apM1 in a dose- and time- dependent manner (146). Administration of TZDs to insulin-resistant humans and mice also increased circulating levels of adiponectin (143).

Although many factors including circulating hormones, therapeutic agents such as PPAR-γ agonists, exercise, and weight loss, have been identified as factors playing a role in the regulation of adiponectin secretion and expression, there are many as yet unidentified factors that also play a role in the regulation of adiponectin. One of these regulatory factors may be the vasoactive peptide, endothelin-1. Endothelin-1 levels are

elevated in obesity and type 2 diabetes, and previous studies demonstrated that endothelin-1 plays a role in the regulation of other adipokines such as leptin and resistin.

Intracellular trafficking of adiponectin

The majority of adiponectin is sorted into a compartment which is secreted constitutively and the remainder is sorted into a regulated compartment. Adiponectin is secreted primarily in a constitutive manner, while insulin and other secretagouges modestly stimulates its secretion (208). Adiponectin forms homotrimers upon translocation into the lumen of the endoplasmic reticulum. The assembly of circulating isoforms of adiponectin begins with a highly stable interaction between the carboxyl-terminal globular domains of three adiponectin subunits and proceeds to the collagenous domain (36). Studies done by Bogan *et al.* showed that some of the adiponectin is retained in the endoplasmic reticulum as its staining overlapped with ER resident protein, GRP94 and the rest of it is in the periphereal storage compartments, probably in the Golgi (16). The importance of Golgi in intracellular trafficking of adiponectin is also highlighted in studies done by Xie *et al.* showing that treatment of adipocytes with Brefeldin A, (a fungal macrolide antibiotic which disrupts the organization of the Golgi complex and prevents vascular budding from the Golgi) inhibits adiponectin secretion. The same study also demonstrated that adiponectin is colocalized with GGA (Golgi localized, γ-ear-containing, Arf binding proteins) proteins in the TGN/Golgi region and its intracellular trafficking and secretion is dependent on GGA-coated vesicles (252). GGA proteins represent a newly recognized, evolutionarily conserved protein family with homology to the "ear" domain of the clathrin adaptor AP-1 γ subunit (18; 265). GGA

proteins may play an important role in cargo-selective clathrin-mediated protein traffic from the *trans*-Golgi network to endosomes (37). A recent study by Clarke *et al.* demonstrated that rab11, a recycling endosomes, is crucial for the secretion of adiponectin as overexpression of mutant form of rab11, Rab11-S25N in 3T3-L1 adipocytes inhibited adiponectin secretion (33). These studies indicate that sorting of adiponectin into cargo vesicles for trafficking to plasma membrane occurs at the level of Golgi via endosomal pathway. Studies done by Bose *et al.,* showed that a v-SNARE Vtila (vps 10p tail interacting 1a), is located in a compartment distinct from the TGN and regulates early step in the trafficking of adiponectin such as budding of these vesicles from their individual compartment (20). These studies indicate that adiponectin may be sorted into different compartment at the Golgi and postGolgi level and trafficking to the plasma membrane may be dependent on different proteins. A recent study by Chen *et al.* showed that chronic ethanol feeding impaired adiponectin secretion by subcutaneous and retroperitoneal adipocytes; impairement of adiponectin intracellular trafficking likely contributes to decreased adiponectin concentrations after chronic ethanol feeding (28). However, the list of proteins or agents involved in intracellular trafficking of adiponectin is far from complete.

Endothelin-1

Discovery of endothelin-1

In 1985, Hickey *et al*. described that endothelial cells released a peptidergic endothelium-derived constricting factor (84). Yanagisawa *et al.* purified and cloned this factor in 1988 (261) and research exploded into the family of endothelin molecules- small

vasoconstrictor peptides produced by the endothelial cells lining vessels walls that these authors described as a family of 21 amino acid peptides named endothelin. Three individual genes encode three different endothelin peptides, and are known as endothelin-1,-2 and -3 (ET-1, ET-2 and ET-3, respectively) (97). The most abundant endothelin peptide in circulation is endothelin-1, which is thought to be constitutively released from endothelial cells. The circulating concentration of ET-1 in plasma is approximately 1 pM (117). Plasma ET-1 is degraded and cleared rapidly, with a half-life of approximately 1 min (6) Under normal physiological conditions, ET-1 is not classified as a circulating hormone, but rather as an autocrine or paracrine factor at many sites of action in the body (117).

Protein structure of endothelin-1

Endothelin-1 (ET-1) is a 21 amino acid peptide and processing of ET1 is the result of a series of proteolytic clevages of the initial gene product (Figure 6). Translation of preproET mRNA generates the peptide preproET. An intermediate form, termed Big Et-1 consisting of 38 amino acid residues, is initially produced from pro-endothelin-1 by a dibasic pair-specific endopeptidase. Big ET-1 is then converted to the mature peptide through proteolytic cleavage by endothelin-convering enzyme (ECE) which cleaves 18 amino acids from the C-terminus of bigET-1 to produce the biologically active peptide ET-1 (Figure 6). Big ET-1 has little vasoactive potency, therefore the conversion of Big ET-1 to ET-1 is considered essential for the effects of ET-1. Although ET-1 is produced primarily from endothelial cells, it is also secreted from a wide variety of other cell types including macrophages, fibroblasts, and cardiomyocytes (255).

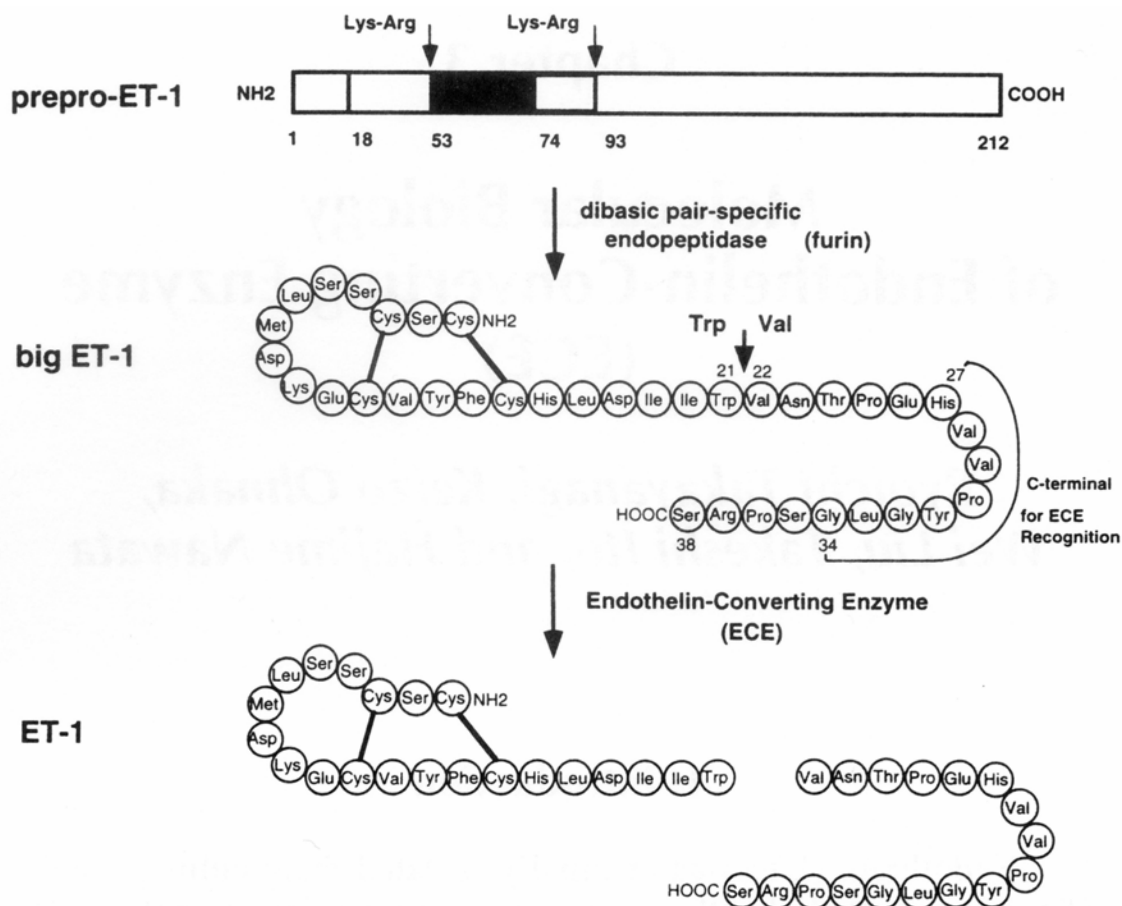

Figure 6. Biosynthetic pathway of human ET-1. Trp^{21} and the sequence from His^{27} and Gly^{34} are important for recognition by ECE. Adapted from Takayanagi R. et al. "Molecular Biology of Endothelin-Converting Enzyme (ECE)". From *Endothelin: Molecular Biology, Physiology, and Pathology.* Edited by R.F. Highsmith @ Humana Press Inc., Totowa, NJ. 1998:76.

Regulation of endothelin-1 expression and secretion

Once ET-1 has been formed, it is secreted primarily via a constitutive pathway. Therefore, ET-1 is regulated at the level of protein synthesis, particularly transcription. Endothelin 1 is secreted predominantly by vascular endothelial cells to act on the underlying smooth muscle cells; however, its secretion also has been shown in several other cells and tissues. ET-1 is secreted primarily toward the basolateral side of the cell. Approximately 80% of the total amount of ET-1 synthesized by endothelial cells is released toward the basolateral side of cells, tissue levels are likely higher than plasma levels. Several regulatory elements are found in the 5′ region of the ET-1 gene (97). The characteristics of the ET-1 gene are similar to the early response genes characterized by rapid up-regulation elicited after a change. Because of this type of regulation and the fact that ET-1 is secreted primarily toward the basolateral side of the cell, plasma levels of ET-1 change slowly in response to diverse stimuli. Therefore, induction of ET-1 secretion above basal levels requires 2 to 5 hours (81; 119). Endothelin 1 plasma levels usually are seen in only the picomolar range (1-10 pmol/L), lower than those required to invoke vasoconstriction. Thus, ET-1 probably acts primarily as a paracrine/autocrine mediator and not as a circulating hormone (238). Vasoconstrictors, such as norepinephrine, angiotensin II (Ang II), vasopressin, F_2-isoprostane, serotonin, oxidized low-density lipoprotein, and transforming growth factor stimulate the synthesis and/or the release of ET-1(45; 126; 261). Vasodilators, such as bradykinin, nitric oxide (NO), prostaglandins E_2 and I_2 , atrial and brain natriuretic factors, and adrenomedullin, often inhibit the release of ET-1 (23; 122; 190).

Endothelin Receptors and Signal Transduction

Endothelins exert their actions via 2 receptor subtypes, ET_A and ET_B, (90; 247) which belong to the super-family of G protein–coupled receptors. Endothelin receptors are widely distributed throughout various tissues including heart, lungs, kidney, and the nervous system (2). The human ET_A receptor, believed to be involved in vasoconstrictive and proliferative responses to ET-1, (119; 238) contains 427 amino acids and binds with the following affinity: ET-1 > ET-2 > ET-3 (90). The human ET_B receptor contains 442 amino acids and binds all ETs with equal affinity; its activation induces transient vasodilation.

ET_A receptors are found in smooth muscle cells of vessels and airways, cardiomyocytes, liver stellate cells, and hepatocytes, as well as brain neurons, osteoblasts, melanocytes, and keratinocytes, adipocytes, and various cell types of the reproductive tract. ET_B receptors are located on vessel endothelial cells and smooth muscle cells, liver hepatocytes and Ito cells, renal-collecting-duct epithelial cells, airway smooth muscle cells, osteoblasts, some neurons, and various cell types of the reproductive tract. Many cell types express both ET_A and ET_B receptor subtypes however ET_A receptors appear to be the predominant receptor subtype of endothelin receptor in many tissues (63; 196; 213).

ET_A receptors were initially cloned from bovine tissues, while ET_B receptors were first cloned from rat tissues (7; 203). Both ET_A and ET_B receptors are members of the G-protein coupled receptor family and induce a signal transduction cascade resulting in phospholipase C (PLC) activation followed by hydrolysis of phosphodatidylinositol 4,5-

bisphosphate (PIP_2) leading to diacylglycerol (DAG) and inositol triphophate (IP_3) generation. IP_3 formation results in an increase in cytosolic calcium leading to smooth muscle contraction. DAG stimulates protein kinase C (PKC) activation leading to mitogenesis and changes in gene expression (Figure 7). It is unclear however if the signal transduction events are the same for each receptor subtype or tissue type. Binding of ET-1 to ET_A receptors also activates receptor-operated and voltage-gated calcium channels in the plasma membrane, allowing entry of extracellular calcium into the cell. This last effect of ET-1, mediated by IP_3 and/or PKC, results in a sustained (minutes) elevation in intracellular calcium, which contributes to the prolonged smooth muscle cell contraction elicited by the peptide (200). Endothelin B coupling activates soluble phospholipase A_2 and guanylate cyclase, leading to an increase in intracellular cyclic guanosine monophosphate. This change stimulates the release of NO and prostaglandin I_2, producing vasodilation (85). Furthermore, ET-1 increases expression of transcription factors, such as c-*fos*, c-*jun*, c-*myc*, and VL-30 (134). Endothelin isopeptides, through either ET_A or ET_B receptors, also induce the mitogen-activated protein kinase cascade (238) that may mediate the long-term action of ET.

Termination of the ET-1 signal upon binding to its receptor is slow to occur due to a very slow rate of dissociation of the protein from its receptor. This slow rate of dissociation explains the prolonged effect of ET-1 on vasoconstriction given its short half-life in the circulation (2). One of the proposed mechanisms of signal termination involves desensitization of the receptor as well as downregulation of many of the second messengers induced by binding of ET-1 to its receptor.

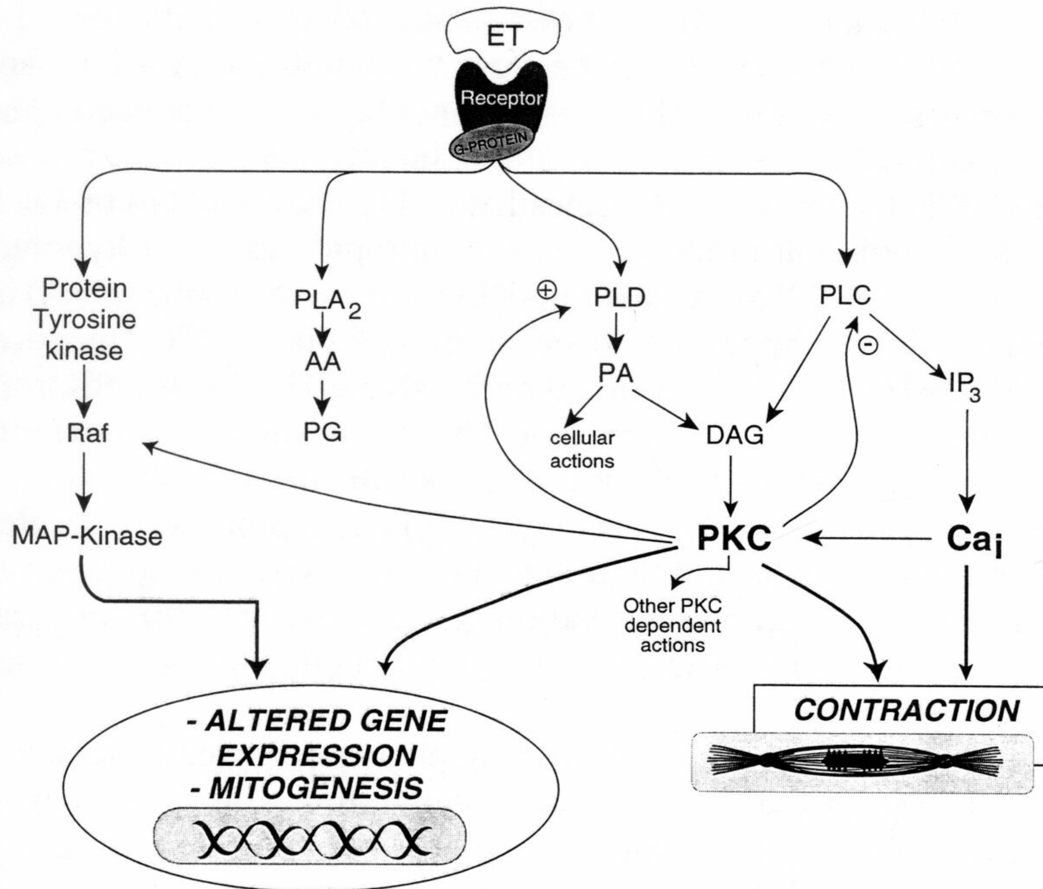

Figure 7. General scheme of ET-1 signal transduction pathways in vascular smooth muscle. ET, Endothelin-1; G protein, guanine nucleotide binding protein; PLC, phospholipase C; IP3, inositol 1,4,5 triphosphate; $Ca_{i,}$ intracellular Ca^{2+} concentration; DAG, diacylglycerol; PKC, protein kinase C; PLD, phospholipase D; PA, phosphatidic acid; PLA_2, phospholipase A_2; AA, arachadonic acid; PG, prostaglandin; PTKs, protein tyrosine kinases; MAP-kinase. Mitogen activated protein kinase. Adapted from Decker E. R. and Brock T. A. "Endothelin Recpetor-Signaling Mechanisms in Vascular Smooth Muscle." From *Endothelin: Molecular Biology, Physiology, and Pathology.* Edited by R.F. Highsmith @ Humana Press Inc., Totowa, NJ. 1998:95.

Physiological functions of ET-1

ET-1 has a large number of physiological functions including a role in the maintenance of vascular tone, sodium balance, neural crest cell development and neurotransmission (117). Intravenous infusion of ET-1 into conscious rats causes an initial decrease in blood pressure that is followed by intense and prolonged (several hours) hypertension (81; 119; 200). The initial decrease in blood pressure apparently is due to activation of the ET_B receptor, which in turn increases release of NO and prostacyclin from the endothelium, atrial natriuretic peptide, and adrenomedullin from adrenal glands (85; 104). The subsequent vasoconstrictive response is due to the direct action of ET-1 on the smooth muscle cells via the ET_A receptor (80; 264). Studies such as these indicate that ET-1 functions as both a vasodilator and as a vasoconstrictor peptide, however its long-term effect on vascular resistance results in it often being classified as a vasoconstrictor peptide. These studies indicate that ET-1 plays a vital role in the maintenance of basal vascular tone.

In the heart, ET-1 affects the coronary circulation, the cardiac myocytes, and the conduction system. ET-1 produces vasoconstrictive responses in human coronary circulation and may play a role in the etiology of coronary vasospasm (14; 136; 202). Furthermore, ET-1 directly enhances platelet aggregation and thrombus formation, which in turn aggravate coronary atherosclerosis and coronary ischemia (153). ET-1 also is a potent mitogen of cardiac myocytes, increasing the release of natriuretic peptides, and has a positive inotropic effect (124). ET-1 plays a major role in regulating renal hemodynamics, tubular handling of water and electrolytes, and proliferation and mitogenesis of mesangial cells and VSMCs (149; 168; 175; 245). ET-1 also has

profound effects on glucose metabolism. Studies have shown that it stimulate hepatic glycogenolysis and increases hepatic glucose output (197; 228). ET-1 also affects glucose uptake, however its effects remain controversial. Wu-Wong *et al*. described a stimulatory effect of ET-1 on glucose uptake in 3T3-L1 adipocytes and neonatal rat cardiomyocytes via activation of the ET_A receptor (251). Ishibashi *et al*. also demonstrated that ET-1 stimulates glucose uptake in 3T3-L1 adipocytes, and that this effect is mediated by GLUT 4 translocation to the membrane (98). However, ET-1 has also been shown to inhibit insulin-stimulated glucose uptake in isolated rat adipocytes and 3T3-L1 adipocytes (30; 135). Idris *et al.* also demonstrated that ET-1 has a transient inhibitory effect on insulin-stimulated glucose uptake in 3T3-L1 adipocytes, however they did not observe any effects of ET-1 on insulin-stimulated glucose uptake in L6 muscle cells (94).

ET-1 also plays a role in the regulation of hormone release from endocrine tissues such as pancreatic islet cells (39; 71) and adipocytes (253; 266). De Carlo *et al.* demonstrated that ET-1 stimulated insulin secretion in a glucose and calcium-dependent manner from isolated rat islets (39). ET-1 has also been demonstrated to effect the secretion of adipokines from adipocytes. Xiong *et al.* demonstrated that ET-1 stimulates leptin expression in Ob-Luc and 3T3-L1, two different adipocyte cell lines (241). The stimulatory effect of ET-1 on leptin secretion was mediated through the ET_A receptor. They also showed that ET-1 positively interacts with insulin to stimulate leptin production. Our laboratory recently demonstrated that ET-1 inhibits basal and insulin-stimulated resistin secretion from 3T3-L1 adipocytes. These findings suggest that ET-1 can affect whole body energy metabolism and insulin sensitivity through the adipocyte-

derived hormones, leptin and resistin. Our laboratory also recently demonstrated that ET-1 acutely stimulates and chronically inhibits adiponectin secretion from 3T3-L1 adipocytes (32).

Role of endothelin-1 in disease

Elevated levels of ET-1 have been reported in numerous pathological states including congestive heart failure, obesity and diabetes (108; 205; 222). It is believed that ET-1 plays a pathological role in the development of cardiovascular disease. Increased circulating levels of immunoreactive endothelins are found in models of experimental heart failure (148). Various investigators have studied the effects of endothelin antagonists in rat models of heart failure and that it can reduce left-ventricular hypertrophy (101) and lower blood pressure (226). Localized increases of ET-1 may also play a role in the development of hypertension, and higher plasma levels of ET-1 have been reported in patients suffering from subarachnoid hemorrhage with cerebral vasospasms (151). ET-1 is also involved in the pathophysiology of myocardial infarction (MI) and congestive heart failure (117). There is some evidence supportive of a role for ET-1 in chronic heart failure. Plasma ET-1 levels increase more than fivefold within a few hours of human myocardial infarction (68). Antagonism of the ET_A receptor decreases the infarct size and is thought to be beneficial in coronary ischemia and reperfusion (72). In a conscious rat model, chronic administration of bosentan, ET_A/ET_B antagonist, significantly reduces arterial blood pressure (226) and decreases cardiac remodeling events following MI and reduces the elevated pulmonary and aortic pressures (164).

Endothelins may also play a role in the development of atherosclerosis. Studies suggest an increase in the production of ET-1 by endothelium may be one of the pathological changes underlying atherosclerosis. In atherosclerotic human subjects, an increase in the circulating levels of ET-1, as well as an increase in immunoreactive ET-1 within blood-vessel may be seen (136; 249). ET-1 released in response to vascular injury is a strong chemoattractant for circulating monocytes, and activates macrophages (76). Macrophages, in turn, can then cause further injury to the overlying endothelium and also produce platelet-derived growth factor (PDGF), interleukin 1, and tumor necrosis factor-α, which can result in secondary up-regulation of PDGF production by smooth muscle cells and endothelium (68; 201). ET-1 has been shown to promote micro vascular platelet thrombus formation (75) and therefore may contribute to acute coronary syndromes.

As in other disease states, plasma concentrations of ET-1 are elevated in states of insulin resistance such as obesity and diabetes, which are characterized by hyperinsulinemia and hyperglycemia (108; 205; 222) and leptin resistance. Plasma concentration of ET-1 is closely associated with insulin secretion and insulin dose in patients with diabetes. Plasma ET-1 is higher in type 2 diabetes than in type 1 diabetes (250). A study conducted by Yamauchi *et al*. showed that elevation of glucose concentrations in cultured media from 5.5 to 11.1 or 22.2 mM significantly stimulated ET-1 release from cultured endothelial cells (259). High glucose has been shown to induce expression of ET-1 in endothelial cells by protein kinase C mechanism (186). Interestingly, leptin increased ET-1 mRNA and protein release from human umbilical vein endothelial cells (HUVECs) in a dose- and time-dependent manner.

Increased secretion of ET-1 from vascular endothelim may contribute to endothelial dysfunction and impairment of insulin signaling. ET-1 increases serine phosphorylation of IRS-1, leading to decreased PI3-Kinase activity in vascular smooth muscle cells (103). Studies suggest that ET-1-mediated vasoconstriction is increased in insulin resistant states such as obesity and diabetes (152). Studies done by Wilkes *et al*. showed that chronic in vivo ET-1 administration leads to in vivo insulin resistance with a decrease in skeletal muscle glucose uptake and inhibition of the insulin signaling pathway (246). Considering the link between these diseases, ET-1 deserves attention with respect to its role in insulin resistance and the control of adipocyte function. Previous clinical studies have demonstrated that ET-1 induces a state of insulin resistance. This phenomenon has also been investigated in cell-based systems and it has been shown that ET-1 prevents insulin-stimulated GLUT4 translocation by adipocytes through the activation of ET_A receptors. This was suggested to involve decreased tyrosine phosphorylation of insulin receptor substrates such as IRS-1(96). Importantly, the reduced insulin-dependent glucose uptake in skeletal muscle in vivo does not result from a vasoconstrictive decrease in skeletal muscle blood flow (176), implying the existence of a direct ET-1 effect on one or more mechanisms involved in insulin-stimulated glucose transport. Recent study done by Strawbridge *et al.* have shown that ET-1–induced insulin resistance results from reversible changes in PIP_2-regulated actin polymerization and not PIP_2-dependent signaling (219). Another study suggested that ET-1 produces a state of insulin resistance in the adipocytes by inhibiting insulin-stimulated GLUT4 translocation via membrane based mechanism (220). The involvement of the PIP_2/actin

system in the adverse effects of ET-1 provide insights into how disturbances in the plasma membrane and cytoskeleton contribute to the development of insulin resistance.

Role of endothelin-1 in adipose tissue physiology

Studies have shown that ET-1 has a dramatic impact on adipose tissue physiology. ET-1 has been shown to affect adipose tissue differentiation (78; 210), to reduce lipoprotein lipase activity and glucose and lipid metabolism in adipocytes (105; 135; 232). ET-1 induces insulin resistance in rat adipocytes by inhibiting insulin-stimulated glucose uptake in rat adipocytes (30) and 3T3-L1 adipocytes . Moreover, studies have shown that ET-1 affects leptin (253), resistin (266) and adiponectin (32) secretion from adipocytes. A recent study has shown a correlation between ET-1 and adiponectin concentrations in humans (183). Besides transcriptional and translational control of protein secretion (253), ET-1 has been shown to affect vesicular trafficking via modulation of actin cytoskeleton. Previous studies have shown that ET-1 induces cortical F-actin assembly through PYK2 (185) and ARF6 and stimulates GLUT4 translocation (19; 131). Strawbridge *et al*. have demonstrated that chronic ET-1 administration disrupts actin cytoskeleton by stimulating PIP_2 hydrolysis via acviation of PLCβ and inhibits insulin-stimulated GLUT4 translocation, thereby inducing insulin resistance in 3T3-L1 adipocytes (219). These findings indicate that ET-1 can directly and indirectly modulate adipocyte functions. Therefore, dysregulation of adipocyte function by ET-1 may disrupt body energy homeostasis and progressively lead to a number of disorders, including type 2 diabetes and cardiovascular disease.

Actin Cytoskeleton

Role of actin cytoskeleton in vesicular trafficking

Over the past several years it has become increasingly apparent that the cell cytoskeleton can have substantial influence over vesicle trafficking events. Most secretory cells have a dense sheet of F-actin beneath and juxtaposed to the plasma membrane, referred to as cortical actin. Several studies have suggested that cortical actin functions as a physical barrier to vesicle docking based upon its transient depolymerization during exocytosis and that secretion preferentially occurs at sites where the actin cortex is relatively thin (236; 237). For example, in chromaffin cells, actin filament networks act as a barrier to the secretory granules, impeding their contact with the plasma membrane (236; 237). Another study demonstrated that cortical F-actin acts as a barrier to prolactin secretion and actin destabilizing agents enhance prolactin secretion in cultured normal anterior pituitary cells (25). In islet beta-cells the predominant function of actin filaments appears to be to provide access of insulin-containing granules to the plasma membrane (137). However, in many cell systems depletion of F-actin structures either by sequestering actin monomers or by stimulation of actin severing does not stimulate exocytosis, but results in an inhibition of agonist-induced secretion (137). For example, pretreatment of HIT-T15 cells with the latrunculin B and cytochalaisn B inhibited stimulated insulin secretion in a time- and dose-dependent manner (137). Catecholamine release from cultured adrenal chromaffin cells is also inhibited by actin disrupting agents (163). Several studies have also suggested a role of cortical actin remodeling in insulin-stimulated GLUT4 translocation in adipocytes (102; 112) as well as in skeletal muscle (227). A rapidly growing body of evidence has

demonstrated that the actin cytoskeleton is important for the regulation and organization of both the exocytic and endocytic functions of the secretory pathway at the level of Golgi/TGN region. Studies have shown roles for the actin cytoskeleton during clathrin-mediated endocytosis for transport at the Golgi apparatus (193). An actin/spectrin/ankyrin cytoskeleton has been implicated in protein transport to and from the Golgi (40). Most importantly, when actin is disrupted with the toxin cytochalasin B, protein transport through the Golgi apparatus is inhibited (86). Studies using immunoelectron microscopy have shown actin, actin-binding proteins, and myosin are bound to Golgi membranes and Golgi-derived vesicles (82; 234). For example, the actin-based motor, Myo1c has been associated with GLUT-4 containing vesicles and is involved in insulin-stimulated GLUT4 translocation in the adipocytes (21). Myosin II has also been shown to be involved in production of constitutive transport of vesicles from the TGN (165). A study showing that disrupting actin has effects on Golgi morphology also provides good evidence that the actin cytoskeleton functions at the Golgi (233). Together, the above studies suggest an important role for the actin cytoskeleton affect in protein trafficking to and from the Golgi apparatus in certain tissues. Several recent studies have suggested a mechanism linking phosphatidylinositol 4,5-bisphosphate (PIP_2) with actin polymerization through the function of N-WASP. Interaction of N-WASP with PIP_2 and activated Rho proteins including Cdc42(198) and TC10 (113) exposes the VCA domain (verprolin homology, cofilin homology, and acidic region), which in turn activates the Arp2/3 complex, resulting in a burst of *de novo* actin polymerization in response to extracellular stimuli (188; 243).

Role of myosin II in vesicular trafficking

The family of unconventional myosins is ever growing and the functions attributed to them seem to expand in parallel. These actin-based motor proteins have been implicated in processes such as endocytosis and exocytosis, the transport of organelles, in spermatogenesis and in neurosensory functions and the regulation of intracellular membrane traffic. The myosin superfamily members share a common N-terminal motor domain which generates movement along actin filaments in an ATP-dependent process. The motor domain is connected to a tail by a flexible neck region. Class II myosins are often termed the 'conventional myosins', the best-studied example of which is skeletal muscle myosin. All other members of the myosin family are called ‘unconventional myosin’ (157). The subfamily of class II contains both muscle and nonmuscle form of myosin II, whose overall structure is similar, consisting of a pair of 200kDA heavy chains which dimerize and assocaite noncovalently with pairs of myosin light chains (17 and 20kDA). The head domain of myosin II heavy chains contain the binding sites for actin and ATP, providing them with actin-activated ATPase activity (29; 162). Two vertebrate heavy chain genes give rise to different isoforms of nonmuscle myosin IIA and IIB which are differentially expressed in cells and tissues (114; 115). While most of the cytokinesis functions of myosin II is cytokinesis as in skeletal muscle, studies have shown that non-muscle myosin are involved in Golgi transport as well. A 200 kD protein found on transport vesicles of the TGN was identified as myosin II and found to be recruited to TGN vesicles in a GTP-dependent, brefeldin A-sensitive manner (95; 165). Myosin II has been shown to be invoved in vesicle transport and fusion in chromaffin cells in the final phases of exocytosis involving transitions affecting the activity of

docked granules (171; 199). Several studies have also shown that myosin II is part of the cytosolic machinery that regulates the budding of constitutive transport vesicles from the Golgi/TGN and is involved in both anterograde and retrograde trafficking at the Golgi (212). A more recent study showed that myosin II plays a novel role in insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (217). Heimann *et al*. showed that both non-muscle myosin II isoforms could be involved in trafficking and that there is highly specific targeting of each protein to distinct vesicle-budding domains of Golgi membranes (82). However the role of myosin II in the intracellular trafficking of proteins in adipocytes remains to be determined.

Hypothesis and Objectives

Adiponectin is an adipocyte-derived hormone with insulin-sensitizing and anti-atherogenic properties. It is the most abundant plasma protein. However, low adiponectin levels are observed in insulin resistant states such as obesity and type 2 diabetes. Adiponectin may provide a link between obesity and the metabolic syndrome. Therefore it is essential to understand the regulation of adiponectin secretion and expression from adipocytes. ET-1 is a 21 amino acid peptide believed to have vasoconstrictor, mitogenic and metabolic properties. ET-1 levels are elevated in metabolic syndromes including obesity, type 2 diabetes and cardiovascular diseases. It is our contention that a critical player in the regulation of adiponectin secretion may be the vasoactive peptide endothelin-1 (ET-1). Recent studies from our laboratory have demonstrated that ET-1 acutely stimulates and chronically inhibits adiponectin secretion from 3T3-L1 adipocytes. The purpose of the present studies is to investigate the

mechanism(s) through which ET-1 regulates adiponectin secretion from 3T3-L1 adipocytes.

Our first series of studies will investigate the chronic effect of ET-1 on adiponectin secretion. These studies will not only provide evidence that ET-1 modulation of actin cytoskeleton through PIP_2 metabolism plays a critical role in adiponectin secretion, but also provide evidence of the importance of actin cytoskeleton in the regulation of adiponectin secretion. Actin cytoskeleton has been implicated in the vesicular trafficking of proteins through the associatin of actin-binding proteins or motors to the vesicles in the Golgi/TGN region. It might be possible that trafficking of adiponectin-containing vesicles is linked to the F-actin through actin-binding proteins which can facilitate translocation of adiponectin-containing vesicles to the plasma membrane and facilitate its tethering to the cortical F-actin and its exocytosis. Myosin II is one of the actin-based motors involved in the constitutive transport of proteins from the Golgi/TGN region. The second set of studies will determine the role of myosin II, an actin-based motor in the intracellular trafficking and secretion of adiponectin from 3T3-L1 adipocytes. These studies will establish a mechanism of how adiponectin is secreted from adipocytes and vascular signals such as ET-1 are capable of dysregulating its secretion, suggesting a vascular-adipocyte axis.

CHAPTER III

ENDOTHELIN-1 INHIBITS ADIPONECTIN SECRETION THROUGH A PHOSPHOTIDYLINOSITOL 4,5-BISPHOSPHATE /ACTIN-DEPENDENT MECHANISM

ABSTRACT

Adiponectin is an adipokine with profound insulin-sensitizing, anti-inflammatory and anti-atherogenic properties. Plasma levels of adiponectin are reduced in insulin resistant states such as obesity, type 2 diabetes, and cardiovascular disease. However the mechanism(s) by which adiponectin concentrations are decreased during disease development is unclear. Studies have shown that endothelin-1 (ET-1), a vasoconstrictor peptide, affects adipocyte glucose metabolism and secretion of adipokines such as leptin, resistin, and adiponectin. The goal of our study was to determine the mechanism by which ET-1 decreases adiponectin secretion. 3T3-L1 adipocytes were treated for 24 hrs with ET-1 (10nM) and then stimulated with vehicle or insulin (100nM) for a period of 1-2 hrs. Chronic ET-1 (24 hr) treatment significantly decreased basal and insulin-stimulated adiponectin secretion by 66% and 47%, respectively. Inhibition of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis by the PLCβ inhibitor, U73122, or exogenous addition of PIP_2: histone carrier (1.25μM: 0.625μM) ameliorated the decrease in basal and insulin-stimulated adiponectin secretion observed with ET-1. However, treatment with exogenous PIP_2: histone carrier complex and the actin depolymerizing agent latrunculin B (20μM) did not reverse the ET-1-mediated decrease

in adiponectin secretion. In conclusion, we demonstrate that ET-1 inhibits basal and insulin-stimulated adiponectin secretion through PIP_2 modulation of the actin cytoskeleton.

INTRODUCTION

The incidence of obesity and type 2 diabetes and the associated risk factors (cardiovascular diseases, dyslipidemia and the metabolic syndrome) continue to increase in the U.S. Obesity and type 2 diabetes are associated with insulin resistance, but the pathological link between them is not fully understood. One link may be adipose tissue, traditionally recognized as an energy storage organ, but now also recognized as an endocrine organ. It secretes various factors “adipokines” such as leptin, resistin, and adiponectin that have been implicated in the development of insulin resistance.

Adiponectin, a 30kDa hormone produced exclusively by white adipose tissue, is one of the most abundant plasma proteins. However, in insulin resistant states such as obesity and type 2 diabetes, its expression and circulating levels are decreased (244). Decreased adiponectin expression and secretion have been positively correlated with a decrease in insulin sensitivity (89). Recently, adiponectin has been recognized to have not only insulin sensitizing properties, but anti-atherogenic properties as well. Adiponectin levels are decreased in patients with coronary heart disease (125) and it has been suggested that

it modulates the endothelial inflammatory state associated with coronary heart diseases (177). Therefore, adiponectin may provide a link between obesity and insulin resistance and understanding the regulation of adiponectin secretion and gene expression is crucial to our increased knowledge of the etiology of insulin resistance. Adiponectin expression and secretion from white adipose tissue is regulated by a variety of factors including tumor necrosis factor-α (TNF-α) (110), interleukin-6 (46), β-adrenergic agonists (43) and insulin (52). However, other factors can also regulate adiponectin secretion, including the vasoconstrictor peptide endothelin-1 (ET-1).

ET-1, a 21 amino acid peptide produced by endothelial cells of the vasculature, has mitogenic and vasoconstrictor properties (109). Circulating levels of ET-1 are elevated in insulin resistant states such as type 2 diabetes (4), cardiovascular disease (127), renal disease (167), and endothelial dysfunction (184). ET-1 induces insulin resistance in rat adipocytes (30) and rat arterial smooth muscle cells (103). In 3T3-L1 adipocytes, it leads to heterologous desensitization of insulin signaling by inhibiting insulin-stimulated GLUT4 translocation (99), thus producing a state of insulin resistance. A recent study has demonstrated ET-1 impairs insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes via a phosphatidylinositol 4,5-bisphosphate (PIP_2)/Actin dependent mechanism (219; 220).

ET-1 also affects adipose tissue protein secretion. ET-1 acutely stimulates leptin secretion from Ob-Luc cells and 3T3-L1 adipocytes (238) and alters resistin secretion from 3T3-L1 adipocytes. Clarke *et al*. demonstrated that ET-1 acutely stimulates and chronically inhibits adiponectin secretion from 3T3-L1 adipocytes (32). PIP_2 has been shown to affect vesicular trafficking and protein secretion (15) through regulation of actin

cytoskeleton, We tested whether PIP_2-modulation of actin polymerization accounts for ET-1's effect on adiponectin secretion.

MATERIALS AND METHODS

Materials. Endothelin-1, latrunculin B and insulin were purchased from Sigma Chemical (St. Louis, MO). All cell culture reagents were obtained from Invitrogen (Grand Island, NY). Adiponectin antibody was purchased from Affinity Bioreagents (Golden, CO). Phosphatidylinositol 4,5-bisphosphate [PIP_2 cat no. P-4516] and histone carrier were purchased from Echelon Biosciences (Salt Lake City, UT). All other reagents obtained from commercial sources were of analytical grade.

Fibroblast Differentiation. 3T3-L1 mouse fibroblasts were purchased from American Type Culture Collection (ATCC-Manassas, VA) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37°C in an atmosphere of 10% CO_2. Two days after confluence was reached, differentiation was induced by incubating the cells for three days in DMEM containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 250 nM dexamethasone and 4 μg/ml insulin. Medium was then replaced with DMEM containing 10% FBS and 4 μg/ml insulin for additional three days. Adipocytes were used 9-12 days post-differentiation. Adipogenesis was monitored by morphological examination of the cells for the accumulation of lipid droplets with Oil Red O (0.2%) staining.

3T3-L1 Adipocyte Treatments. Adipocytes were treated with 10 nM ET-1 for 24 hrs. For inhibitor treatments, 3T3-L1 adipocytes were pretreated with 1 μM ET_A-R antagonist, BQ-610, and 20 μM PLCβ inhibitor, U-73122 or respective vehicle for 30 min and then incubated with ET-1 with or without antagonists or inhibitors (10 nM) for 24 hrs. After 24 hrs, cells were serum starved for 3 hrs, treated with 20 μM latrunculin B (60 min) hr or with PIP_2: histone complex (30 min) and stimulated with insulin (100 nM) or vehicle for 1 hr. Media was taken at designated times and adiponectin in the media was measured by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (10%) and immunoblotting. Briefly, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in TBS-T containing 7% non-fat dried milk, incubated overnight with murine adiponectin polyclonal antibody (1:1000) (Affinity Bioreagents, Golden, CO), washed, and incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000). Blots were developed using ECL chemiluminescence reagents (Amersham Biosciences, England). The intensities of the resultant bands (optical pixel density) were determined using the BioRad Fluor-S Multimager System.

Adiponectin Gene Expression. RNA from 3T3-L1 adipocytes was extracted with TRIzol (Invitrogen, Carlsbad, CA). One ug total RNA was reverse transcribed using iScript cDNA Synthesis kit with 100 U of Superscript II Reverse Transcriptase (BioRad, Hercules, CA). Adiponectin mRNA expression was measured by quantitative real-time PCR (iCycler iQ Real-Time PCR detection System, BioRad, Hercules, CA). Two μL of each RT reaction was amplified in a 30 μL PCR reaction containing 200 μM of each

primer and SYBR Green Super Mix (BioRad, Hercules, CA). Samples were incubated in the iCycler for an initial denaturation at 95°C for 3.0 min followed by 40 PCR cycles. Each cycle consisted of 95°C for 10 s and 58°C for 1 min. The following oligonucleotide primers were used: adiponectin (Accession No. U37222) AGTTTGAGAGTCCTGAGTATTATCC(sense), TGTTATTGCTACGATGTAAGAGT (antisense); 36B4 (Accession No.NM007475) AAGCGCGTCCTGGCATTGTCT (sense) and CGCAGGGGGCAGCAGCAGTGGT (antisense). SYBR Green I fluorescence emission was measured after each cycle. Adiponectin mRNA levels were normalized to 36B4 expression. Amplification of specific transcripts was confirmed initially by sequencing and subsequently by producing melting curve profiles each real-time PCR run (cooling the sample to 55°C and heating to 95°C with continuous measurement of fluorescence).

Whole-cell Immunoflorescence and Phalloidin Staining. 3T3-L1 adipocytes after 8 day of differentiation were detached from culture dishes with 0.25% trypsin and reseeded in chamber slides. Cells were allowed to recover for 24 hrs before treatments. Following treatment, adipocytes were fixed for 20 min at 25°C in 2% paraformaldehyde/Tris-buffered saline (PIP_2 labeling) or 4% paraformaldehyde/0.2% Triton X-100/PBS (actin labeling). For labeling of PIP_2 after fixation, cells were incubated in 0.1% Triton X-100/ Tris-buffered saline (TBS) for 20 min at 25°C, blocked for 60 min with 5% donkey serum in TBS and incubated for 1 hr with 1:100 dilution of mouse anti-PI 4,5-P_2 antibody (Assay Designs, Ann Arbor, MI). For labeling of actin after fixation, cells were incubated with Alexa-fluor 488 conjugated phalloidin for 20 min at 25°C. Samples were

examined with a Nikon microscope. Images were made with Q imaging camera and Q capture Pro Software. All microscopic and camera settings were identical within experiments, and representative images are shown. All images were processed and fluorescence intensity was measured in Adobe Photoshop.

Statistical Analysis. Data from all experiments are expressed as means ± standard error of the mean (SEM). Statistical analyses were performed using one-way ANOVA and differences between groups determined by the Tukey-Kramer multiple comparisons test ($P < 0.05$).

RESULTS

Chronic ET-1 treatment inhibits basal and insulin-stimulated adiponectin secretion. 3T3-L1 adipocytes were treated with ET-1 (10 nM) for 24 hrs and then were stimulated with vehicle or insulin (10 nM) for 1 hr. ET-1 inhibited basal and insulin-stimulated adiponectin secretion by 66% and 47%, respectively (Fig. 8). No changes in intracellular adiponectin concentrations or cell viability were observed during chronic treatment with ET-1 (data not shown). ET-1 treatment reduced insulin-stimulated adiponectin secretion to a basal level suggesting a differential regulation of constitutive and regulated adiponectin-containing compartments as previously suggested in studies done by Bogan *et al*. (16).

BQ-610 prevents ET-1-mediated decrease in basal and insulin-stimulated adiponectin secretion. 3T3-L1 adipocytes possess only the ET_A receptor and ET-1's effect on leptin

(253), resistin secretion and GLUT4 translocation in 3T3-L1 adipocytes are mediated through this receptor. Therefore, we determined if ET-1's inhibitory effect on adiponectin secretion is ET_A receptor-mediated. Cells were treated with or without ET-1 in the presence or absence of ET_A receptor antagonist, BQ-610. Incubation with BQ-610 prevented the ET-1-mediated decrease in basal and insulin-stimulated adiponectin secretion completely (Fig.9). In the absence of ET-1, BQ-610 had no effect on basal or insulin-stimulated adiponectin secretion (Fig. 9).

Chronic ET-1 treatment does not effect adiponectin gene expression. After observing that ET-1 inhibits basal and insulin-stimulated adiponectin secretion, it was important to determine if this effect was transcriptional or posttranscriptional. Mature 3T3-L1 adipocytes were treated with insulin (100 nM) or ET-1 (100 nM) for 1-24 hrs and adiponectin gene expression was analyzed using quantitative real-time RT-PCR. Insulin significantly downregulated adiponectin gene expression compared to control within 16 hrs of treatment (Fig. 10A), however ET-1 did not alter adiponectin gene expression at any time point studied (Fig. 10B). In order to investigate a possible concentration-dependent effect of insulin or ET-1 on adiponectin gene expression, mature 3T3-L1 adipocytes were treated with insulin (0.01-100 nM) or ET-1 (0.01-100 nM) for 16 hrs. Insulin (10 nM and 100 nM) significantly downregulated adiponectin gene expression (Fig. 10C); however ET-1 had no effect on adiponectin gene expression at any concentration evaluated (Fig. 10D). These studies demonstrate that ET-1 inhibition of adiponectin secretion is through posttranscriptional regulation, and suggest that chronic

ET-1 signaling may have a pronounced effect on adiponectin packaging and/or vesicular trafficking.

Inhibition of PIP_2 hydrolysis and PIP_2 addition prevent/restore decreased adiponectin secretion due to chronic ET-1 treatment. Studies have shown that chronic ET-1 stimulation of PLCβ leads to loss of plasma membrane PIP_2. Consistent with the studies done by Strawbridge *et al*. (219), we demonstrated that immunofluorescence PIP_2 labeling was greatly diminished in ET-1 treated cells as compared to controls (Fig. 11A). Exogenous addition of PIP_2 via carrier mediated delivery restored PIP_2 in ET-1 treated cells (Fig. 11A).

Next we evaluated whether inhibition of PIP_2 hydrolysis or addition of exogenous PIP_2 restores the ET-1-mediated decrease in adiponectin secretion. Treatment of 3T3-L1 adipocytes with the phospholipase Cβ (PLCβ) inhibitor, U-73122 (10μM) prevented the decrease in basal adiponectin secretion observed after chronic treatment with ET-1 (Fig. 12). This effect was also observed with insulin-stimulated adiponectin secretion. We also demonstrated that carrier-mediated delivery of exogenous PIP_2 (1.25μM) in ET-1-treated cells recovers the basal and insulin-stimulated adiponectin secretion (Fig. 13). These data suggest that the presence of adequate concentrations of PIP_2 in the plasma membrane are critical for the trafficking of adiponectin-containing secretory vesicles.

Cortical F-Actin polymerization is an important step in adiponectin secretion. PIP_2 regulates actin dynamics in 3T3-L1 adipocytes . A decrease in PIP_2 levels at the plasma membrane results in a decrease in F-actin polymerization (27). Chronic ET-1 treatment

results in a decrease in cortical F-actin as shown by phalloidin staining in fixed cells (Fig. 14). To determine whether chronic ET-1 treatment inhibits adiponectin secretion by depolymerization of the actin network, it was necessary to first demonstrate that adiponectin secretion is an actin-mediated event. 3T3-L1 adipocytes were pretreated with the actin depolymerizing toxin, latrunculin B (20μM) for 1 hr and then stimulated with vehicle or insulin for 1 hr. Latrunculin B significantly inhibited basal and insulin-stimulated adiponectin secretion by 63% and 40%, respectively (Fig. 15). Interestingly, insulin was able to partially stimulate adiponectin secretion even when F-actin remains impaired by Latrunculin B. These studies suggest that actin network is not a barrier, but is necessary for constitutive adiponectin secretion; however, regulated secretion of adiponectin is independent of intact cortical F-actin, at least in part. As mentioned previously, studies have demonstrated that PIP_2 stimulates actin polymerization, which is important for the movement of specific vesicles to the cell surface. We determined whether the significant restoration in basal adiponectin secretion by PIP_2 was dependent on F-actin by evaluating the effect of PIP_2 in conjunction with latrunculin B (Fig. 16). Consistent with PIP_2 being associated with F-actin regulation, replenishment of PIP_2 did not restore basal adiponectin secretion in the presence of latrunculin B (Fig. 16). These studies suggest that ET-1 inhibits constitutive adiponectin secretion by depolymerizing the actin network indirectly through PIP_2 depletion, thereby impairing the adiponectin secretory pathway. However, insulin-regulation of adiponectin secretion appears to be independent of F-actin.

DISCUSSION

Even though adiponectin was only recently discovered, studies have demonstrated that it is directly involved in a number of disease states. Originally, it was observed that adiponectin plasma concentration and gene expression are reduced in obesity and type 2 diabetes (244). Recently, it has also been demonstrated that adiponectin has inflammatory-modulating activities, and clinical studies have demonstrated inverse associations between adiponectin levels and serum markers of inflammation (180). Adiponectin involvement in a variety of disease state suggests that a number of factors may be involved in the regulation of adiponectin secretion. It is our contention that one of these factors is ET-1.

Studies have demonstrated that ET-1 has a dramatic impact on adipocyte physiology. ET-1 inhibits adipocyte differentiation (78), reduce lipoprotein lipase activity, impact glucose metabolism in adipocytes (232) and synthesis and release of adipokines such as leptin , resistin and adiponectin (32). These findings indicate that ET-1 can directly and indirectly modulate adipocyte functions. Therefore, dysregulation of adipocyte functions by ET-1 may disrupt whole body energy homeostasis and progressively lead to a number of disorders, including type 2 diabetes and cardiovascular diseases.

Our studies demonstrate that chronic ET-1 treatment inhibits basal and insulin-stimulated secretion of adiponectin secretion in 3T3-L1 adipocytes, thereby targeting both constitutive and regulated compartments. Its effect on adiponectin secretion is not based on modulation of gene transcription but is mediated by effects on vesicular trafficking by depleting plasma membrane PIP_2. Chronic ET-1 treatment leads to PIP_2 depletion from the plasma membrane (220) which correlates with ET-1-induced decrease

in adiponectin secretion. Prevention of ET-1-mediated PIP_2 hydrolysis by the PLC β inhibitor, U-73122 or PIP_2 replenishment restores adiponectin secretion.

The mechanism by which PIP_2 regulates adiponectin secretion likely involved the actin cytoskeleton. Mature adipocytes possess a layer of cortical actin filament, primarily at the plasma membrane. Cortical F-actin may be a barrier for vesicles to fuse to the plasma membrane (132) or can be a scaffold necessary for trafficking of some vesicles . PIP_2 modulates actin dynamics in 3T3-L1 adipocytes (111) and its loss leads to dysregulation of cortical F-actin (27). While studies have demonstrated cortical F-actin to be important for regulated insulin-stimulated translocation of GLUT4, our studies demonstrate that the actin cytoskeletal integrity is also crucial for constitutive secretion of adiponectin. Maintenance of this cortical F-actin is dependent on PIP_2, which is present at the plasma membrane in basal states. Insulin stimulation further enhances F-actin polymerization (92).

Adiponectin is primarily located in the Golgi and its intracellular trafficking is dependent on GGA-coated vesicles (252). Its path from the Golgi to the plasma membrane is not known. Our study demonstrates that F-actin is important for adiponectin secretion; most likely functioning as a scaffold for adiponectin-containing vesicles to fuse to the plasma membrane. The failure of PIP_2 add back to restore adiponectin secretion in the presence of latrunculin B in ET-1-treated cells highlights the importance of F-actin in adiponectin secretion. A recent study by Clarke *et al*. demonstrating that adiponectin is secreted in an rab11/ARF6-dependent manner (33) further supports our theory that F-actin forms a "road" for adiponectin secretion, as ARF6 has been shown to regulate F-actin polymerization (120) .

The majority of adiponectin is sorted into a compartment which is secreted constitutively and the remainder is sorted into a regulated compartment (16). Adiponectin is secreted primarily in a constitutive manner, while insulin and other secretagouges modestly stimulate its secretion (208). Stimulation of the regulated compartment by insulin is PI3K- and Akt-dependent (110). The ability of insulin to stimulate adiponectin secretion in latrunculin B-treated cells suggests that the regulated compartment of adiponectin-containing vesicles is F-actin independent. Although latrunculin B treatment depolymerizes F-actin, it does not affect insulin-stimulated Akt phosphorylation in 3T3-L1 adipocytes (112). Studies have shown that insulin-mediated F-actin polymerization and PI3K activation are independent of each other in 3T3-L1 adipocytes (102), suggesting that PI3K/Akt activation can mediate trafficking of vesicles irrespective of actin cytoskeleton integrity. Consistent with this, insulin was able to stimulate adiponectin secretion even when F-actin remains impaired by latrunculin B probably due to insulin-stimulated PI3K and Akt activity. These observations are supported by a study done by Eyster *et al.*, showing that constitutively active PKB/Akt signals GLUT4 translocation in the absence of intact actin cytoskeleton (47), which further suggest that insulin stimulates adiponectin secretion by enhancing mobilization of adiponectin-containing vesicles of regulated compartment. This regulated secretion is Akt-dependent and is not affected by actin depolymerization. These studies indicate a differential regulation of constitutive and regulated secretion of adiponectin. Previous studies have shown that chronic ET-1 treatment impaired Akt-1 activity, but not Akt-2 activity in 3T3-L1 adipocytes. Chronic ET-1 treatment did not impair the ability of

insulin to stimulate adiponectin secretion, suggesting the possibility of insulin-stimulated adiponectin secretion to be Akt-2-dependent.

These studies also point to the important fact that actin cytoskeleton plays an important role in the trafficking and secretion of adiponectin. Golgi actin plays an important role in the membrane vesicle transport through the association of actin-binding proteins or motors to the vesicles (66; 67; 70; 113). It is be possible that trafficking of adiponectin-containing vesicles is linked to the F-actin through actin-binding proteins which can facilitate translocation of adiponectin-containing vesicles to the plasma membrane and facilitate its tethering to the cortical F-actin and its exocytosis. Studies have shown that regulation of insulin-stimulated GLUT4 translocation by actin cytoskeleton is via actin-based motor, myo1c (21; 22). Since adiponectin secretion is primarily a constitutive process, it is possible that actin-based motors that are associated with transport of constitutive vesicles might be involved in trafficking of adiponectin. One such motor is myosin II that has been shown to be involved in the production of constitutive transport vesicles from the TGN (165). Further studies are needed to investigate the importance of F-actin and myosin II in the exocytosis process of adiponectin-containing vesicles.

While our understanding of regulation of adiponectin secretion and its decrease in insulin-resistant states is limited, importance of membrane lipids and cytoskeleton in adiponectin secretion may advance our knowledge. Not only do these studies indicate how adiponectin secretion is regulated, they also point to the fact that vascular signals such as ET-1 are capable of dysregulating its secretion, suggesting a vascular-adipocyte axis.

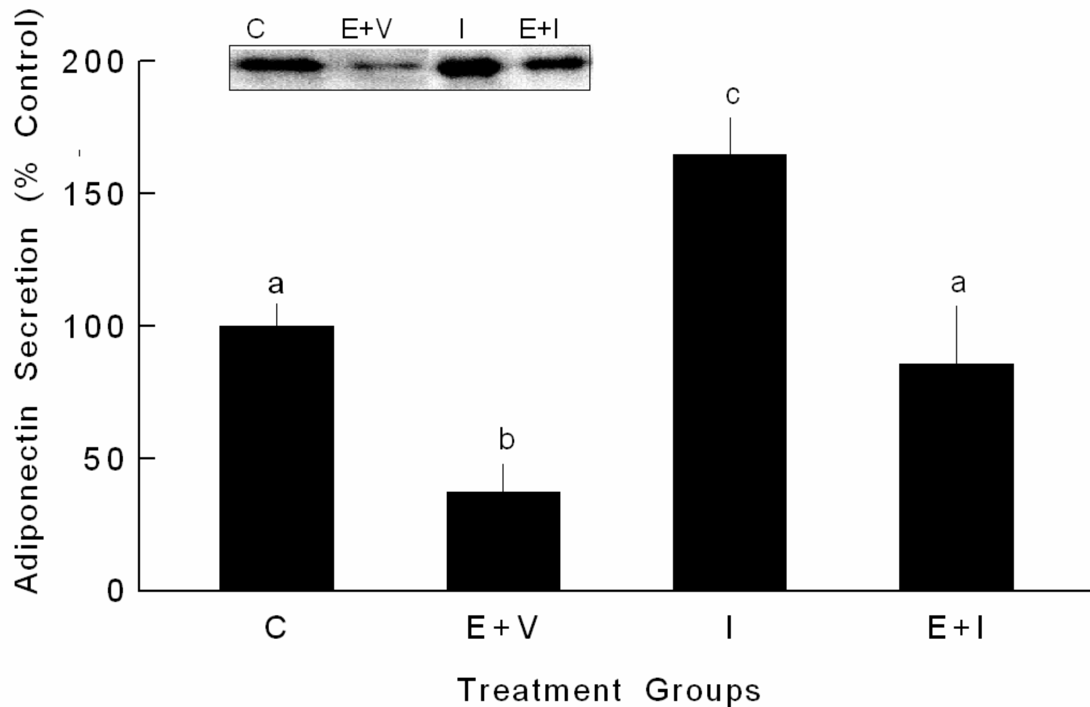

Figure. 8. Chronic ET-1 inhibits basal and insulin-stimulated adiponectin secretion. 3T3-L1 adipocytes were treated with endothelin-1 (E) (10 nM) for 24 hrs. After serum starvation, adipocytes were stimulated with vehicle (v) or insulin (I) (10 nM) for 1 hr. Adiponectin secretion was measured from the media using SDS-PAGE and immunoblotting. Data are expressed as mean ± SEM of 3 separate experiments. Values with different letters are significantly different ($P < 0.05$). A representative blot is shown at the upper portion of the graph.

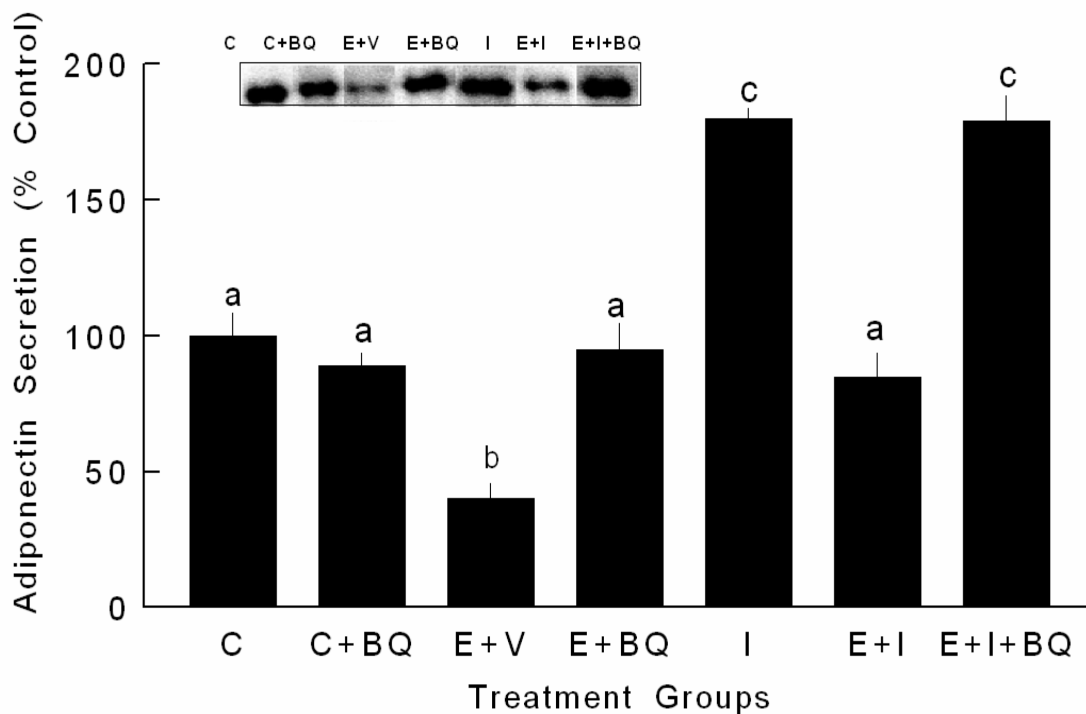

Figure 9. BQ-610 prevents ET-1-mediated decrease in adiponectin secretion. 3T3-L1 adipocytes were pretreated with the ET_A receptor inhibitor BQ-619 (BQ) (1 μM) for 30 min and then treated with endothelin-1 (E) (10 nM) for 24 hrs. After serum starvation, adipocytes were stimulated with vehicle (v) or insulin (I) (10 nM) for 1 hr. Adiponectin secretion was measured from the media using SDS-PAGE and immunoblotting. Data are expressed as mean ± SEM of 3 separate experiments. Values with different letters are significantly different ($P < 0.05$). A representative blot is shown at the upper portion of the graph.

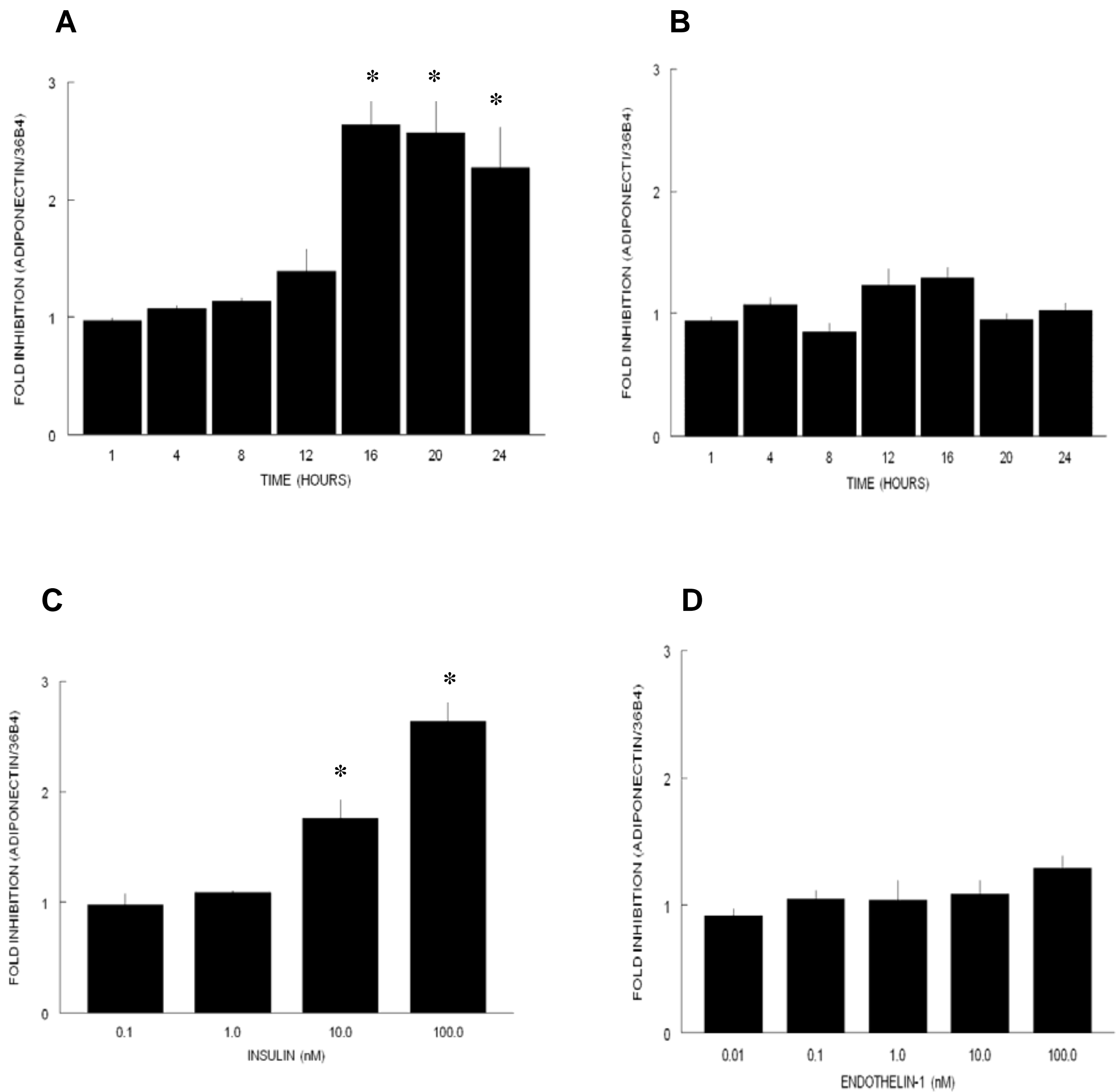

Figure 10. <u>ET-1 does not regulate adiponectin gene expression in a time- or concentration-dependent manner.</u> 3T3-L1 adipocytes were serum-starved for 3 hours then treated with insulin (100nM) or ET-1 (100 nM) for 1-24 hours (2A, 2B). In a separate series of exp eriments, 3T3-L1 adipocytes were treated with insulin (0.1-100 nM) or ET-1 (0.01-100 nM) for 24 hrs (2C, 2D). Adiponectin gene expression was analyzed by real-time RT-PCR. Bars represent means ± SEM of 3 separate experiments and are shown as fold inhibition (adiponectin/36B4). ∗ $p \leq 0.05$ vs. control.

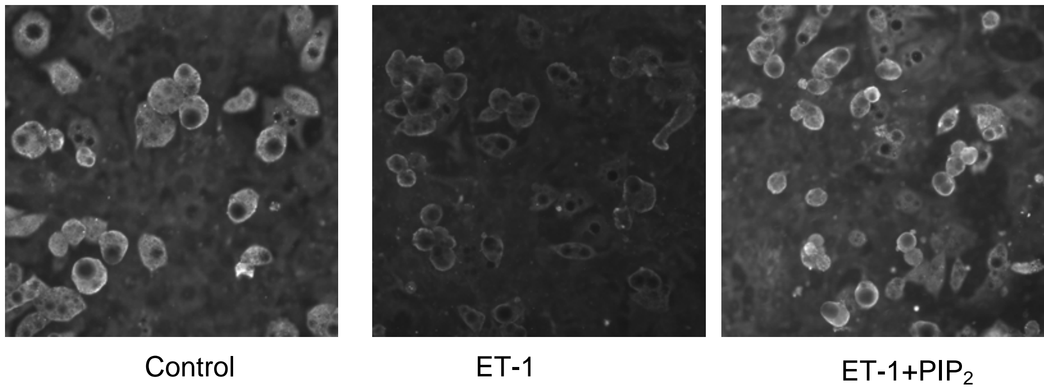

Figure 11. Chronic ET-1 treatment decreases PIP_2 in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with ET-1 (10nM) for 24 hrs, serum starved for 3 hrs and PIP_2: histone complex was added back for 1hr and PIP_2 labeling detected in the cells by immunoflourescence.

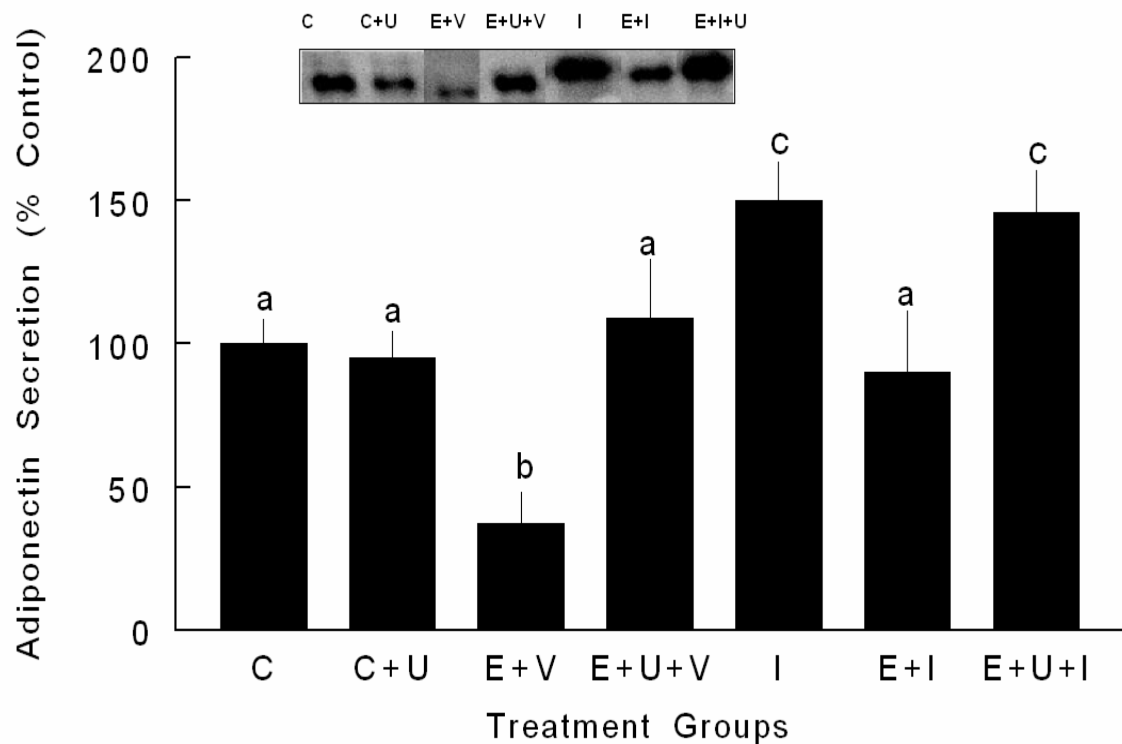

Figure 12. U73122 prevents ET-1-mediated decrease in basal and insulin-stimulated adiponectin secretion. 3T3-L1 adipocytes were pretreated with the PLCβ inhibitor, U73122 (U) (10μM) for 30 min and then treated with endothelin-1 (E) (10nM) for 24 hrs. After serum starvation, adipocytes were stimulated with vehicle (v) or insulin (I) (10 nM) for 1 hr. Data are expressed as means± SEM from 3 separate experiments. Values with different letters are significantly different ($P < 0.05$). Representative blots are shown at the upper portion of the graphs.

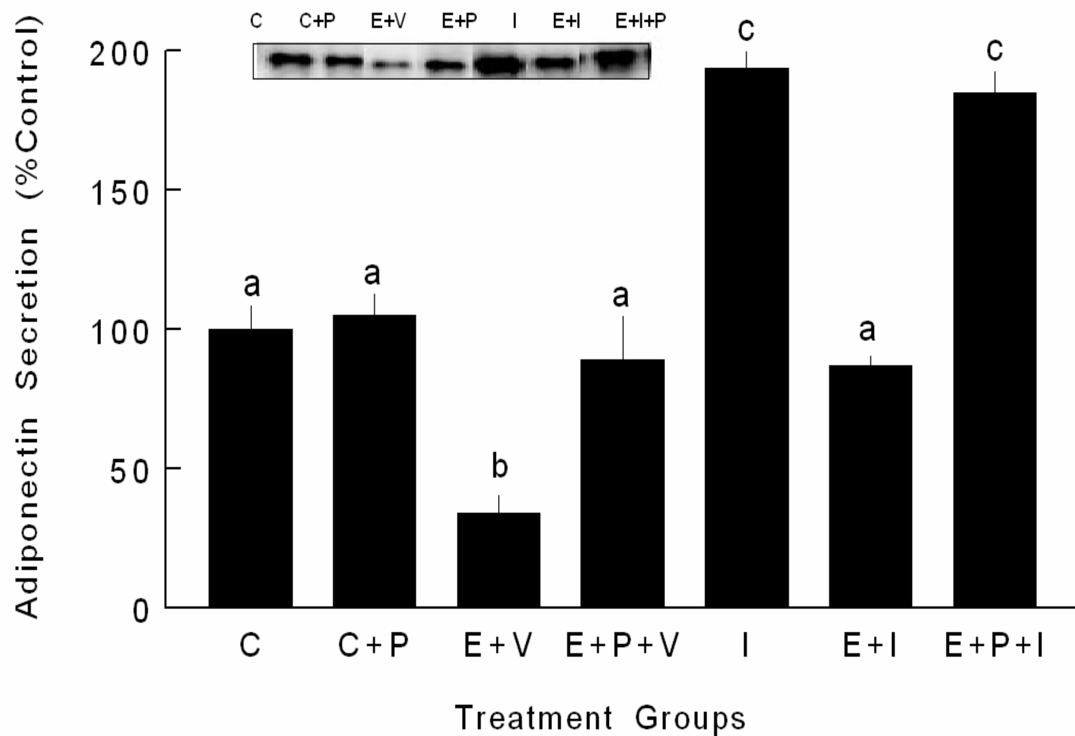

Figure 13. PIP$_2$ addition restores the basal and insulin-stimulated adiponectin secretion in ET-1 treated cells. 3T3-L1 adipocytes were treated with endothelin-1 (E) (10nM) for 24 hrs. After serum starvation, PIP$_2$: Histone complex (P) (1.25μM: .625μM) was added for 30min and then stimulated with vehicle (v) or insulin (I) for 1 hr. Images and data are expressed as means± SEM from 3 separate experiments. Values with different letters are significantly different ($P < 0.05$). Representative blots are shown at the upper portion of the graphs.

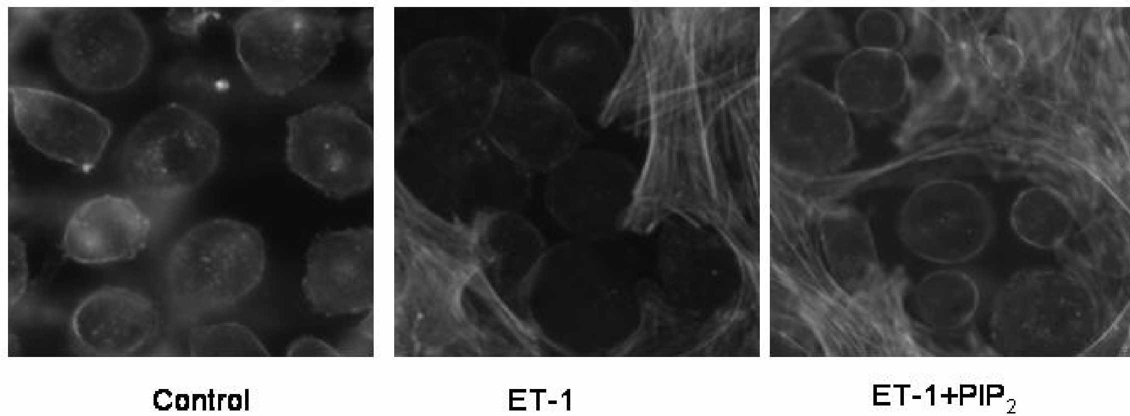

Figure 14. Chronic ET-1 treatment decreases cortical F-actin in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with ET-1 (10nM) for 24 hrs, serum starved for 3 hrs and PIP_2: histone complex was added back for 1hr and actin staining detected by immunofluorescence. Image expressed as means± SEM from 3 separate experiments.

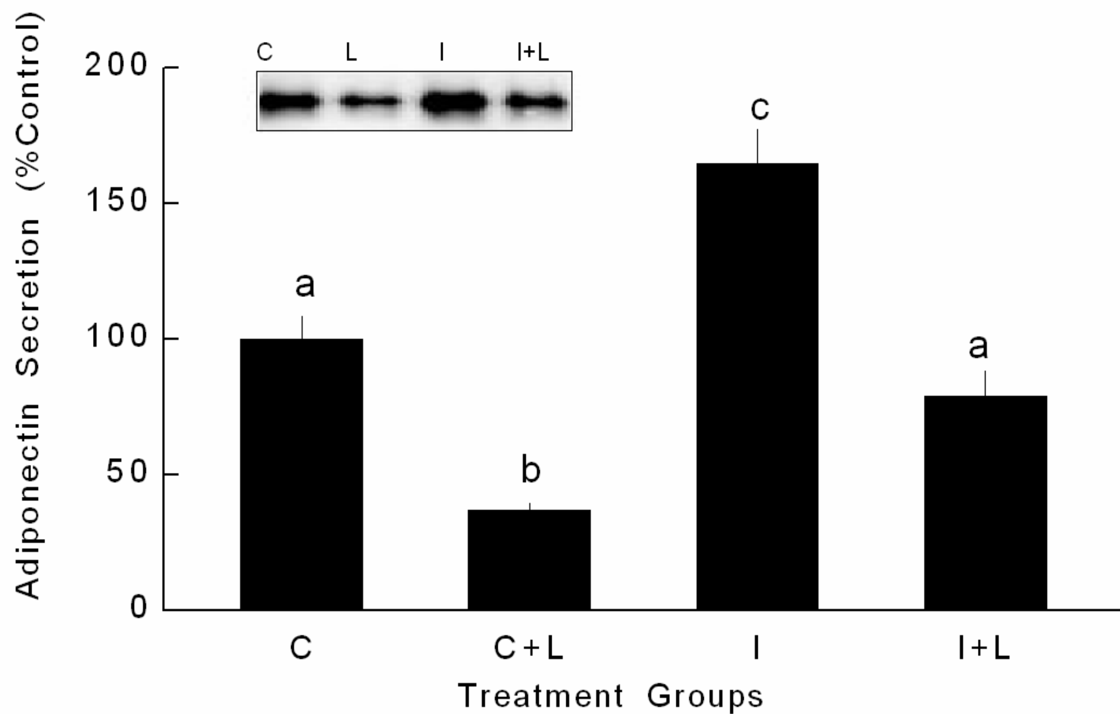

Figure 15. Latrunculin B inhibits adiponectin secretion. 3T3-L1 adipocytes were pretreated with latrunculin B and then stimulated with vehicle or insulin for 1 hr. Data are expressed as mean ± SEM from of 3 separate experiments. Values with different letters are significantly different ($P < 0.05$). Representative blots are shown at the upper portion of the graphs.

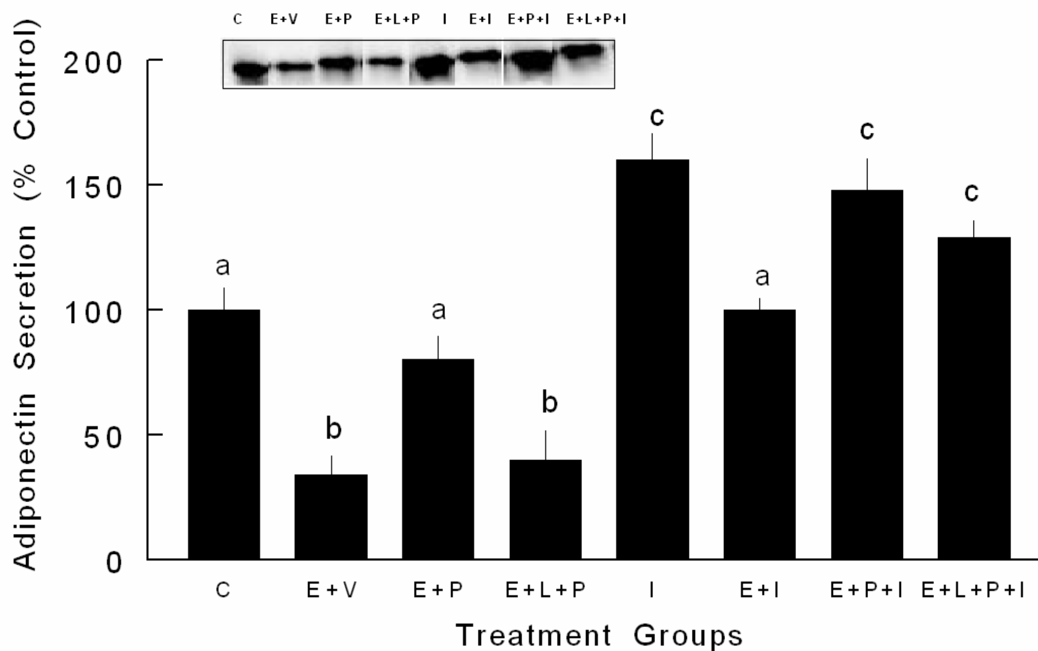

Figure 16. PIP$_2$ does not restore adiponectin secretion in ET-1-treated cells when actin remained impaired with latrunculin B. 3T3-L1 adipocytes were treated with endothelin-1 (E) (10nM) for 24 hrs. After serum starvation, pretreated with latrunculin B (L) for 1 hr and then treated with PIP$_2$: Histone complex (P) (1.25µM: .625µM) for 30 min and stimulated with vehicle (V) or insulin (100nM) (I) for 1hr. Data are expressed as mean ± SEM from of 3 separate experiments. Values with different letters are significantly different ($P < 0.05$). Representative blots are shown at the upper portion of the graphs.

CHAPTER IV

REGULATION OF INTRACELLULAR TRAFFICKING AND SECRETION OF ADIPONECTIN BY MYOSIN II

ABSTRACT

Adiponectin is an adipokine secreted exclusively by white adipocytes which plays an important role in insulin action, energy homeostasis and the prevention of atherosclerosis. The mechanisms responsible for adiponectin trafficking and secretion are not completely understood. We investigated the role of myosin II, an actin-based motor, in the trafficking and secretion of adiponectin in 3T3-L1 adipocytes. Myosin IIA and IIB isoforms were colocalized with adiponectin as determined by immunofluorescence. Immunogold electron microscopy of purified adiponectin-containing vesicles also provided evidence that myosin IIA and IIB were present in adiponectin-containing vesicles. Immunofluorescent and immunogold microscopy revealed that myosin IIA and IIB were dispersed throughout the cytoplasm of the cell. Both myosin isoforms were localized in the Golgi/TGN region as evidenced by colocalization with the cis-Golgi marker, p115 and the TGN marker, γ-adaptin. Inhibition of myosin II activity by blebbistatin or actin depolymerization by latrunculin B dispersed myosin IIA and IIB towards the periphery, while significantly inhibiting adiponectin secretion. Therefore, the constitutive trafficking and secretion of adiponectin in 3T3-L1 adipocytes appears to

occur by an actin-dependent mechanism that involves the actin-based motors, myosin IIA and IIB.

INTRODUCTION

Adipose tissue is not only considered an energy storage organ, but also an endocrine organ that secretes a variety of factors including "adipokines" such as leptin, resistin, adiponectin, and adipsin (62). Adiponectin (Acrp30) is an adipokine secreted exclusively by white adipocytes with insulin-sensitizing (34; 140), anti-atherogenic (116; 178) and anti-inflammatory properties (44; 58). Although it is one of the most abundant serum proteins, adiponectin expression and plasma concentrations are decreased in obesity and type 2 diabetes (8). Adiponectin expression and secretion have been positively correlated with insulin sensitivity (89).

Although the intracellular localization and trafficking of leptin, adipsin, and the glucose transporter, GLUT4, have been well studied, little is known regarding the intracellular trafficking of adiponectin. Unlike GLUT4 and leptin which are mostly insulin-regulated, adiponectin is secreted primarily in a constitutive manner. Previous studies have shown that the majority of adiponectin is sorted into vesicles that undergo constitutive secretion, while a small portion is sorted into regulated secretory vesicles (16). Adiponectin is localized in the endoplasmic reticulum (16) and peripheral storage vesicles in the Golgi/TGN region (252). Recent studies have demonstrated that endoplasmic reticulum ER oxidoreductase Ero1-Lalpha and effectors modulating PPARgamma and SIRT1 activity regulate secretion of adiponectin from 3T3-L1 adipocytes (192) and is covalently linked to ER chaperons ERp44 that controls

posttranslational events controlling adiponectin secretion (242). Proper Golgi structure and function appear to be crucial for adiponectin secretion since the dispersion of Golgi by brefeldin A inhibits adiponectin secretion (252). Recent studies have shown that there is an abundant pool of properly folded adiponectin in the secretory pathway that is retained by thiol-mediated retention (242). Adiponectin appears to be colocalized with the Golgi marker, p115 and the TGN marker, syntaxin 6 (252). According to a recent investigation, GGA proteins are involved in the trafficking and secretion of adiponectin (252). Adiponectin secretion is also dependent on the recycling of endosomes containing rab-11 (33) and the SNARE protein Vtila (20). However, the list of proteins involved in adiponectin secretion is far from complete.

Protein trafficking through the Golgi complex requires a series of membrane-bound vesicles or tubules. Each vesicle population has a distinct set of coat complexes involved in selective trafficking of cargo proteins. The Golgi complex is associated with diverse cytoskeletal structures and various Golgi-derived vesicles which carry multiple molecular motors to interact with cytoskeletal structures (56; 218). Actin cytoskeletal elements are also involved in Golgi trafficking. In adipocytes, studies have shown that insulin-dependent GLUT4 translocation is dependent on cortical F-actin and insulin-regulated F-actin polymerization (102). A recent study from our laboratory has shown that constitutive adiponectin secretion is dependent on PIP_2 levels and the integrity of the cortical F-actin (11).

Actin-based myosin motors are involved in the vesicular transport of Golgi-derived vesicles in many cells (169; 170). Brush border myosin I has been localized in vesicles budding from the Golgi and secretory granules (57; 161; 187). Unconventional

myosin, myo1c has been identified in GLUT-4 containing vesicles in adipocytes and is responsible for the insulin-stimulated fusion of GLUT-4 to the plasma membrane (21). Myosin 5a interacts with actin as well as microtubules and helps in synaptic vesicle transport, movement of the smooth endoplasmic reticulum and pigment granules (157; 189). Non-muscle myosin II has been shown to be associated with membrane vesicles and participates in vesicular trafficking in mammalian cells (55). Previous studies have shown that myosin II mediates the release of vesicular stomatis viral G protein (VSVG) transport vesicles from the TGN of MDCK cells (165). Several studies have also shown that myosin II is part of the cytosolic machinery that regulates the budding of constitutive transport vesicles from the Golgi/TGN and is involved in both anterograde and retrograde trafficking at the Golgi. A more recent study showed that myosin II plays a novel role in insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (217).

The aim of this investigation was to explore the role of myosin II in the intracellular trafficking and secretion of adiponectin. We demonstrate that inhibition of myosin II activity with blebbistatin inhibits adiponectin secretion. Furthermore, myosin IIA and IIB are associated with adiponectin-containing membranes and may be involved in the intracellular trafficking of adiponectin.

MATERIALS AND METHODS

Materials. Rabbit polyclonal myosin IIA antibody, blebbistatin and brefeldin A were purchased from Sigma (St. Louis, MO). Rabbit polyclonal myosin IIB was purchased from Covance (Princeton, NJ). Rabbit polyclonal and mouse monoclonal adiponectin antibodies were obtained from Affinity Bioreagents (Golden, CO). Mouse monoclonal

p115, γ-adaptin, calreticulin, and Na/KATPase were purchased from BD Biosciences (San Jose, CA). Alexa-fluor 488 donkey anti-mouse, Alexa-fluor 488 donkey anti-rabbit and Alexa-fluor 594 donkey anti-mouse were from Invitrogen (Carlsbad, CA) and gold conjugates for immunogold electron microscopy were from Jackson Immunoresearch (West Grove, PA).

Cell Culture. 3T3-L1 mouse fibroblasts were purchased from American Type Culture Collection (ATCC-Manassas, VA). Fibroblasts were grown and differentiated as described previously (18).

Subcellular Fractionation and Sucrose Gradient Centrifugation. 3T3-L1 adipocytes were grown in 150 mm dishes. On day eight of differentiation, 3T3-L1 adipocytes were treated with either latrunculin B (20 μM) or vehicle for 2 hr or with blebbistatin (100 μM) or vehicle for 3 hrs. Adipocytes were washed twice with ice-cold HES buffer (20 mM HEPES, 1mM EDTA, 250 mM sucrose) and scraped with a rubber policeman in HES supplemented with a protease inhibitor cocktail (Roche Diagnostics, New York, NY). The cells were homogenized and subjected to a differential centrifugation procedure to prepare the plasma membrane and heavy and light microsome fractions as previously described (74). LDM contained the Golgi and endosomal markers. HDM contained dense material including the ER. Further fractionation of the LDM was performed as described previously with minor modifications (31). Briefly, LDM (2 mg) was resuspended in 2 mL of 20 mM HEPES, 100 mM NaCl and 1 mM EDTA, loaded onto a 10–35% sucrose gradient, and centrifuged in a SW 41 Ti rotor at 24,000 rpm for 3.5 h.

Fractions (500 μL) were collected from the top of the tube and analyzed for adiponectin, myosin IIA and myosin IIB. Peak fractions containing these proteins were combined and sedimented at 210,000 g for 3 hrs. The resulting pellet was resuspended in 20 mM HEPES, 100 mM NaCl, and 1 mM EDTA, loaded onto a 10–60% sucrose gradient for equilibrium density centrifugation and centrifuged at 30,000 rpm in an SW 41 Ti rotor for 19 h. Fractions (500 μL) were collected from the top of the tube and stored at −80°C prior to analysis.

Immunoblotting. Adiponectin was analyzed by using SDS–PAGE (10%) and immunoblotting. Briefly, proteins separated by SDS–PAGE were electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in LI-COR odyssey blocking buffer for 1 hr, incubated overnight with rabbit anti-adiponectin polyclonal antibody (1:500) or rabbit anti-myosin IIA (1:500), anti-myosin IIB (1:500), mouse anti-calreticulin (1:1000) or mouse anti-Na/KATPase monoclonal antibody (1:250). Membranes were washed with PBS/0.1% Tween-20 three times and incubated with infrared conjugated secondary antibodies (1:20,000). Blots were scanned using a LI-COR Odyssey Infrared Scanner (Lincoln, NE).

Immunofluorescence Microscopy Whole-cell Immunofluorescence. 3T3-L1 adipocytes were detached from culture dishes with 0.25% trypsin and reseeded in chamber slides. Cells were allowed to recover for 24 h before treatments. Following treatment with blebbistatin (100 μM) for 3 hrs or latrunculin B (20 μM) for 1 hr, adipocytes were fixed for 20 min at 25°C in 4% paraformaldehyde/0.2% Triton X-100/PBS. Cells were

incubated in 0.1% Triton X-100/PBS for 20 minutes at 25°C, blocked for 30 minutes with 5% donkey serum in PBS and incubated for 1 h with, 1:100 mouse anti-adiponectin, 1:200 dilution of rabbit anti-myosin IIA and IIB antibody, 1:100 mouse anti-p115 or 1:100 mouse anti-γ-adaptin. Chamber slides were washed three times with PBS and incubated with anti-mouse Alexa-fluor 488 or anti-rabbit Alexa-flour 594 for 60 minutes at 25°C. Samples were mounted with vectasheild (Vector Laboratories, Burlingame, CA) and examined with a Nikon microscope. Images were made with a Q imaging camera and Q capture Pro Software. All microscopic and camera settings were identical within experiments and representative images are shown. All images were processed in Adobe Photoshop.

Immunoelectron Microscopy. Immunolocalization *of Myosin IIA and IIB in Cultured Adipocytes.* Adipocytes were fixed for 20 minutes in 2% paraformalin/PBS at room temperature. After brief buffer rinses, cells were permeabilized in 0.2% Triton-X-100/PBS for 30 minutes; residual aldehydes were quenched using 0.1% sodium borohydride/PBS for 10 minutes and 0.05 M glycine/PBS for 30 minutes. Antigen retrieval was accomplished using 1 mM EDTA/PBS, pH 8.0 for 30 minutes at room temperature. Cells were blocked prior to primary antibody incubation using 3% BSA, 1% normal donkey serum, plus 0.1% cold water fish skin gelatin (Electron Microscopy Sciences, Fort Washington, PA) in PBS for 30 minutes. Cells were then washed in acetylated BSA (BSA-C) (Electron Microscopy Sciences, Hatfield, PA) incubation buffer (0.1% BSA-C/PBS) followed by incubation in rabbit anti-myosin IIA and IIB beta myosin diluted 1:10 in incubation buffer and held at 4°C for approximately 48 hours.

Following incubation buffer rinses, samples were labeled using donkey α-rabbit 6 nm gold conjugate (Electron Microscopy Sciences, Hatfield, PA) diluted 1:40 in 0.5% BSA-C/PBS for 90 minutes at room temperature. Following incubation buffer washes, one 5 minute high salt (750 mM NaCl) PBS wash was performed to aid in the removal of non-specifically bound gold label. After subsequent PBS rinses and post-fixation in 2% glutaraldehyde/PBS, adipocyte culture samples were dehydrated, infiltrated and embedded in Durcupan AMC Fluka (Electron Microscopy Sciences, Hatfield, PA) according to the manufacturer's recommendations. Ultrathin (75 nm) sections were cut using a Reichert Ultracut S (Leica, Deerfield, Illinois), counter stained with aqueous 2% uranyl acetate and 0.4% lead citrate and viewed with a Philips 301 transmission electron microscope at an accelerating voltage of 60 kV.

Immunolabeling of Isolated Adipocyte Membrane Preparations. Adiponectin and myosin IIA and IIB-containing membranes from equilibrium density gradient fractions were isolated as described above. Fractions were pooled and pelleted at 39,000 rpm for 3 hr, resuspended in PBS and processed for double labeling as described by Martin *et al*. (150). Briefly, freshly prepared adipocyte membrane suspensions were fixed with an equal volume of 2% paraformalin/PBS. Without removing the fixative, 10 µL of the mixture was pippetted on to 150 mesh, formvar coated nickel grids (Electron Microscopy Sciences, Hatfield, PA). The suspension was allowed to dry and grids were sequentially floated on 35 µL drops of 0.02 M glycine/PBS, twice for 5 minutes each, followed by 10 minutes on drops of 0.5 M ammonium chloride/PBS, then 5 minutes on drops of 0.1% BSA/PBS.

Grids were then suspended upon drops of primary antibody blocker containing 3% BSA, 1% normal donkey serum, and 0.1% cold water fish skin gelatin/PBS for approximately 1 hour. Grids were washed 3 times for 5 minutes each in BSA-C incubation buffer (0.1% BSA-C/PBS). The entire panel of primary antibodies (below) were diluted with incubation buffer 1:10 (volume/volume). Grids were floated on mixtures of equal volume (20 μL) of anti-adiponectin, anti-myosin IIA, anti-myosin IIB, anti-p115 and anti-γ-adaptin overnight at 4°C. Two grids were incubated with diluent only as negative controls. Following primary incubation, samples were allowed to reach room temperature and were washed 6 times for 5 minutes each on drops of BSA-C incubation buffer. Donkey anti-rabbit 6 nm gold conjugate was diluted 1:25 with 0.1% BSA/PBS. Donkey anti-mouse 15 nm gold conjugate was diluted 1:20. Equal volumes of the diluted conjugates were mixed into single 20 μL drops upon which each of the 8 grids was suspended and incubated for 2 hours at room temperature. Grids were washed 4 times for 5 minutes each with 0.1% BSA/PBS, followed by 3 times 5 for minutes each in PBS. Label was stabilized with 2% glutaraldehyde/PBS, followed by PBS and distilled water washes. All grids were counter-stained with aqueous 2% phosphotungstic acid for approximately 1 minute and examined with a Philips 301 transmission electron microscope at an accelerating voltage of 60 kV.

Statistical Analysis. Data from all experiments are expressed as means ± standard error mean (SEM). Differences were determined using Student's independent t-test ($p < 0.05$). All statistical analyses were conducted using JMP software.

RESULTS

Subcellular localization of myosin IIA, IIB and adiponectin. The distribution of myosin IIA and IIB within adipocytes was examined using standard subcellular fractionation of adipocytes to yield PM, HDM, LDM and cytosolic fractions. Myosin IIA was present in all membrane fractions except the cytosolic fraction and the highest levels were detected in the LDM fraction. Myosin IIB was also present in all fractions except cytosol with the highest level detected in LDM (Fig. 17 and (31)). Adiponectin was primarily present in the HDM and PM fractions, but was also detectable in the LDM and cytosolic fractions (Fig. 17).

Myosin IIA and IIB localization was also determined by immunofluorescence and electron microscopy in 3T3-L1 adipocytes. Immunofluorescence microscopy revealed that myosin IIA (Fig. 18B, *left*) and IIB (Fig. 18B, *right*) staining was dispersed throughout the cytoplasm while exhibiting some perinuclear localization. Immunoelectron microscopy of adipocytes further confirmed that myosin IIA (Fig. 18C, *left*) and IIB (Fig. 18C, *right*) was scattered throughout the cytoplasm.

Colocalization of adiponectin with myosin IIA and IIB. To determine if myosin IIA and IIB were associated with adiponectin-containing membranes, membranes were purified from 3T3-L1 adipocytes on a 10-35% sucrose gradient by velocity gradient centrifugation of the LDM fractions. Adiponectin exhibited a broad range of distribution indicative of a heterogeneous population of vesicles (Fig. 19A). Interestingly, myosin IIB showed a similar distribution with considerable overlap. Myosin IIA had a narrow range of distribution but also overlapped with adiponectin (Fig. 19A). Fractions

containing adiponectin membranes (1-5 and 13-19) from sucrose velocity gradient were pooled and subjected to (10-60%) sucrose equilibrium gradient centrifugation to further purify the membranes. Most of the adiponectin was enriched in fractions 10-18 after this procedure (Fig. 19B). Myosin IIB was also highly enriched in these fractions and its peak coincided with adiponectin (Fig. 19B). Myosin IIA was present in fractions 11-15 and also colocalized with adiponectin. These studies indicate that adiponectin, myosin IIA and myosin IIB colocalizes in 3T3-L1 adipocytes.

These studies were confirmed with immunofluorescent and immunogold electron microscopy of 3T3-L1 adipocyte staining with adiponectin, myosin IIA and IIB antibodies. Immunofluorescent analysis showed that myosin IIA (Fig. 20, *left, upper*) and IIB (Fig. 20, *left lower*) staining was present throughout the cytoplasm. Adiponectin staining was observed in the perinuclear region and throughout the cytoplasm as shown (Fig. 20, *center, upper and lower*) (252). Staining for both isoforms overlapped with adiponectin, mostly in the perinuclear region (Fig. 20, *right, upper and lower*).

Immunoelectron microscopy was performed on membrane preparations containing adiponectin fractions obtained from the equilibrium gradient centrifugation using anti-myosin IIA, IIB and adiponectin antibodies. Most of the membranes were reactive with anti-adiponectin (15 nM gold) and myosin IIA (Fig. 21, *left*) and IIB (6 nM gold) (Fig. 21, *right*). These studies demonstrate that myosin IIA and IIB interact with adiponectin in 3T3-L1 adipocytes.

Colocalization of myosin IIA and IIB with cis-Golgi marker, p115 and TGN marker, γ-Adaptin. To examine if myosin IIA and IIB resides in the Golgi/TGN region, we

performed colocalization studies against the cis-Golgi marker, p115 and TGN marker, γ-adaptin. Immunofluorescent analysis showed that myosin IIA (Fig. 22A and D) and IIB (Fig. 22G and J) was dispersed throughout the cytoplasm as shown earlier, while staining for p115 (Fig. 22B and H) and γ-adaptin (Fig. 22E and K) was confined to the perinuclear region near the Golgi. Staining of anti-myosin IIA, myosin IIB and p115 overlapped significantly in the perinuclear region (Fig. 22C, 22I).

To further confirm the colocalization of these proteins, immunoelectron microscopy was performed on membrane preparations containing myosin IIA and IIB fractions obtained from equilibrium density centrifugation using anti-myosin IIA, IIB and anti-p115 antibodies. A fraction of myosin IIA (Fig. 23A) and myosin IIB (Fig. 23B) containing membranes colocalized with p115. These studies suggest that both myosin IIA and IIB are present in the Golgi region.

Immunofluorescent analysis of myosin IIA with γ-adaptin (Fig. 22I) only partially overlapped with each other while there was a considerable amount of overlapping between myosin IIB and γ-adaptin in the perinuclear region (Fig. 22DL). Immunogold labeling of membranes isolated from the equilibrium gradient centrifugation also showed that vesicles labeled for myosin IIA and γ-adaptin (Fig. 23C) were mostly separate and only a fraction of these membranes were adjacent to each other. Fractions of membranes labeled for myosin IIB and γ-adaptin colocalized with each other (Fig. 23D).

Blebbistatin inhibits adiponectin secretion and redistributes myosin IIA and IIB. Blebbistatin is a specific myosin II inhibitor which binds to myosin II and decreases actin-activated myosin ATPase activity and thus affects its affinity to bind to actin (123).

However, it does not inhibit myosin I, V or X (5; 141). Blebbistatin has been shown to inhibit both isoforms of myosin II (32). Treatment of 3T3-L1 adipocytes with 100 μM blebbistatin significantly inhibited adiponectin secretion by 65% (Fig. 24A). Subcellular fractionation of adipocytes treated with vehicle or blebbistatin revealed that myosin IIA and IIB were decreased in the LDM fractions and increased in the cytosolic fraction after treatment with blebbistatin (Fig. 24B). Adiponectin was substantially increased in the HDM and LDM fractions (Fig. 24B) after treatment with blebbistatin. Immunofluorescence examination of myosin IIA and IIB in blebbistatin untreated (Fig. 24C, *left*) or treated adipocytes (Fig. 24C, *right*) showed their dispersion from the perinuclear region to the periphery suggesting that the actin-myosin association is necessary for myosin IIA and IIB binding to Golgi membranes.

Latrunculin B inhibits adiponectin secretion and redistributes myosin IIA and IIB. Since myosin IIA and IIB are actin-based motors and latrunculin B has been shown to disrupt F-actin (38), we examined the effect of latrunculin B on myosin IIA and IIB localization. As previously reported by our group (18), latrunculin B decreased adiponectin secretion by 65% (Fig. 25A). Examination of the subcellular fractionation of adiponectin in latrunculin-B treated cells showed high levels of adiponectin in the HDM fractions but low levels in the PM (Fig. 25B) while myosin IIA and IIB localization did not change. However, immunofluorescent microscopy of latrunculin-B treated cells showed reduced myosin IIA (Fig. 25C, *upper*) and IIB signals (Fig. 25C, *lower*) from the perinuclear site with increased peripheral dispersion.

DISCUSSION

Adiponectin plays a central role in the regulation of energy metabolism and homeostasis owing to its effects on energy expenditure and fatty acid oxidation (64; 230). Adiponectin also possesses insulin-sensitizing, anti-inflammatory and anti-atherogenic properties. Low circulating levels of adiponectin are associated with type 2 diabetes, obesity and cardiovascular disease and is considered a biomarker of the metabolic syndrome (9). Therefore, therapeutic interventions designed to raise adiponectin levels may reduce the risk of metabolic and cardiovascular disease.

Despite the association between low adiponectin levels and metabolic and cardiovascular disease conditions, little is known about the intracellular trafficking and secretion of adiponectin. Bogan *et al*. showed that adiponectin is retained in the endoplasmic reticulum and the rest of it is in the peripheral storage compartments, probably in the Golgi (16). More studies have shown that GGA proteins interact with adiponectin-containing vesicles in the Golgi region and play an important role in the intracellular trafficking and secretion of adiponectin (252). Golgi membranes and Golgi-derived vesicles are associated with many cytoskeletal proteins and motors. Studies have shown that association of actin and actin-binding proteins with Golgi membranes are important in vesicular trafficking (66; 67; 70; 82). Recent studies from our laboratory demonstrated an important role of F-actin in the secretion of adiponectin. Another study by Clarke *et al*. suggested a role of ARF6 in the regulation of adiponectin secretion (32). ARF6 has been shown to regulate membrane traffic and remodel F-actin (120; 206). To further elucidate the mechanism by which actin cytoskeleton is important in the constitutive secretion of adiponectin, we designed studies to investigate the role of

myosin II, an actin based motor, in the intracellular trafficking and secretion of adiponectin.

A number of studies have described the role of myosin II in vesicular trafficking (199; 204; 217; 248). Myosin II has been reported to be involved in production of constitutive transport vesicles in the TGN (165) where it colocalizes with VSVG on MDCK Golgi membranes and is involved in the release of VSVG vesicles from TGN. Other studies have shown that myosin II label distinct set of vesicles in the Golgi region (82). Previous studies have shown that myosin IIA is associated with Golgi membranes (96) and is involved in vesicle budding (15). Myosin IIB has been shown to be associated with the plasma membrane (217) as well as the Golgi (82). Therefrore, the present study was designed to examine the intracellular distribution of myosin IIA and IIB and their role in adiponectin trafficking and secretion. Immunofluorescent and immunogold electron microscopy in our studies revealed that myosin IIA and IIB are dispersed throughout the cytoplasm and perinuclear region while subcellular fractionation reveals that both isoforms are present in all adipocytes fractions except cytosol suggesting membranous protein nature of myosin IIA and IIB.

These studies were further confirmed by electron microscopy of myosin IIA and IIB membranes fractionated by equilibrium density centrifugation. Myosin IIA and IIB containing membranes colocalized with p115, a protein involved in vesicular trafficking in the Golgi, as shown by immunofluorescence and immunogold microscopy in 3T3-L1 adipocytes. p115 has been shown to colocalize with perinuclear actin (113) and our findings that myosin IIA and IIB colocalize with p115 suggest that myosin II may be a link between Golgi-derived vesicles or Golgi membranes and F-actin as an actin-binding

protein involved in the vesicular trafficking of proteins in the TGN/Golgi region of adipocytes. Some of the myosin IIA-containing membrane colabelled for γ-adaptin, a component of clathrin-coated vesicles, although the majority of myosin IIA-containing membranes were individually labeled suggesting that myosin IIA is localized on small, non-clathrin-coated vesicles as previously observed (82). The majority of myosin IIB-containing membranes colabelled with γ-adaptin, suggesting that myosin IIB is localized in clathrin-coated vesicles which is consistent with other studies (82). These studies confirm that more than one population of Golgi-derived vesicles or membranes are associated with myosin II and that myosin II isoforms are targeted separately to specific vesicle/membranous populations. These studies are in consistent with other studies demonstrating that nonmuscle myosins IIA and IIB have been found on distinct membrane domains and have been ascribed to different cellular functions such as cytokinesis and cell motility (118; 155). Our findings are also consistent with previous studies (82) which show that myosin II is associated with Golgi/TGN derived clathrin and non-clathrin coated vesicles/membranes and may represent transport vesicles for separate steps of protein transport. It has been suggested that the surfaces of Golgi-derived vesicles appear to be associated with multiple classes of motors, perhaps for a series of temporal interactions with microfilaments (82). One of the possible mechanisms for binding of the Golgi vesicles to actin filaments, and their associated proteins, include direct binding via myosins I or II or indirect association of actin with the vesicle surface through spectrin/ankyrin assemblies.

Previous studies have shown that adiponectin has perinuclear localization and colocalizes with the cis-Golgi marker, p115 and the TGN marker, syntaxin 6 (252). To

examine whether the perinuclear localization of myosin IIA and IIB colocalized with adiponectin, we used three approaches. 1) Sucrose velocity centrifugation and equilibrium density centrifugation showed that the peak of adiponectin-containing membranes coincided with myosin IIB and to a lesser extent myosin IIA. 2) Immunofluorescent microscopy demonstrated that adiponectin colocalizes with myosin IIA and IIB in the perinuclear region. 3) Electron microscopy of vesicles isolated from the equilibrium density centrifugation showed that a fraction of the adiponectin-positive membranes colocalized with myosin IIA and IIB. Myosin II has been shown to be involved in the constitutive transport of vesicles from the TGN (165), while chromaffin cell secretion is dependent on the activity of the myosin II motor (199). A recent study by Suzuki *et al*. (221) has demonstrated that myosin II is involved in the secretion of cyclophilin A in vascular smooth muscle cell. Our studies clearly demonstrate that myosin II is an integral part of adiponectin-containing membranes and is involved in the intracellular trafficking and secretion of adiponectin.

Several studies support the hypothesis that actin is necessary for maintaining Golgi structure (133) and the use of actin-polymerizing and depolymerizing agents have served to implicate the role of the actin cytoskeleton in vesicular trafficking (83; 211). Previous studies have demonstrated that myo1c, actin-based motor, is present on GLUT-4 containing vesicles and is involved in the insulin-stimulated translocation of GLUT4 to the plasma membrane and facilitating its tethering to the actin filaments (21; 22). Studies have shown that disruption of the normal Golgi actin correlates with changes in secretory membrane protein transport in adipocytes (43). These studies provide supporting evidence that an actin-binding protein such as myosin II may be linked to Golgi vesicular

trafficking in adipocytes. Blebbistatin, a specific myosin II inhibitor, significantly inhibited adiponectin secretion while increasing adiponectin levels in the HDM and LDM fractions and dispersed both myosin II isoforms from the LDM fraction to the cytosol. These results suggest that blebbistatin affects myosin IIA and IIB binding to the Golgi membrane which is essential for the translocation of adiponectin-containing membranes to the plasma membrane and its exocytosis. As suggested earlier, myosin II may be involved in the secretion of adiponectin possibly by moving actin filaments to either transport vesicles away from the donor membrane or by allowing actin rearrangements in the vicinity of the Golgi cytoskeleton thus allowing vesicular budding. Another possible mechanism might involve translocation of myosin IIA and IIB with the adiponectin membranes to the plasma membrane where they functions to facilitate membrane fusion to the cortical actin and directing exocytosis of adiponectin.

Previous studies have shown the effect of latrunculin B, an actin depolymerizing agent, on myo1c subcellular distribution (21). Similarly, or studies also shows that latrunculin B dispersed myosin IIA and IIB from the perinuclear region to the periphery. Latrunculin B also inhibited adiponectin secretion while increasing its concentration in the HDM and LDM fractions. These results suggest that F-actin is important for the recruitment of myosin IIA and IIB to the Golgi membranes as suggested by previous studies and for adiponectin secretion confirming our blebbistatin studies.

In summary, our studies indicate a novel role of myosin II in the intracellular trafficking and secretion of adiponectin. Future studies will provide additional details in the vesicular trafficking of adiponectin which may also hold true for other adipokines.

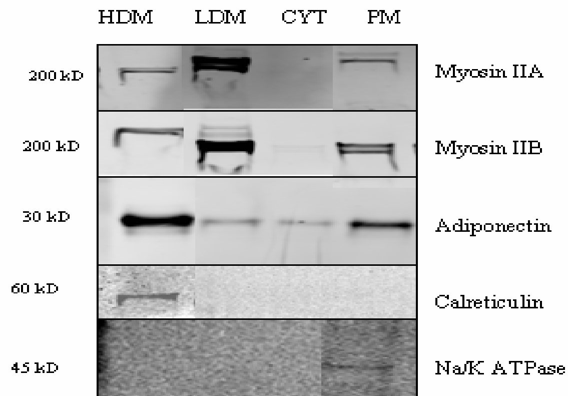

Figure 17. Localization of myosin IIA, IIB and adiponectin. 3T3-L1 adipocytes were homogenized and separated by differential centrifugation into four subcellular fractions: HDM, LDM, CYT and PM. Myosin IIA, IIB and adiponectin protein content were detected by Western blotting using equal quantities of protein (5 μg). The protein markers for the ER and PM are calreticulin and Na/K ATPase, respectively.

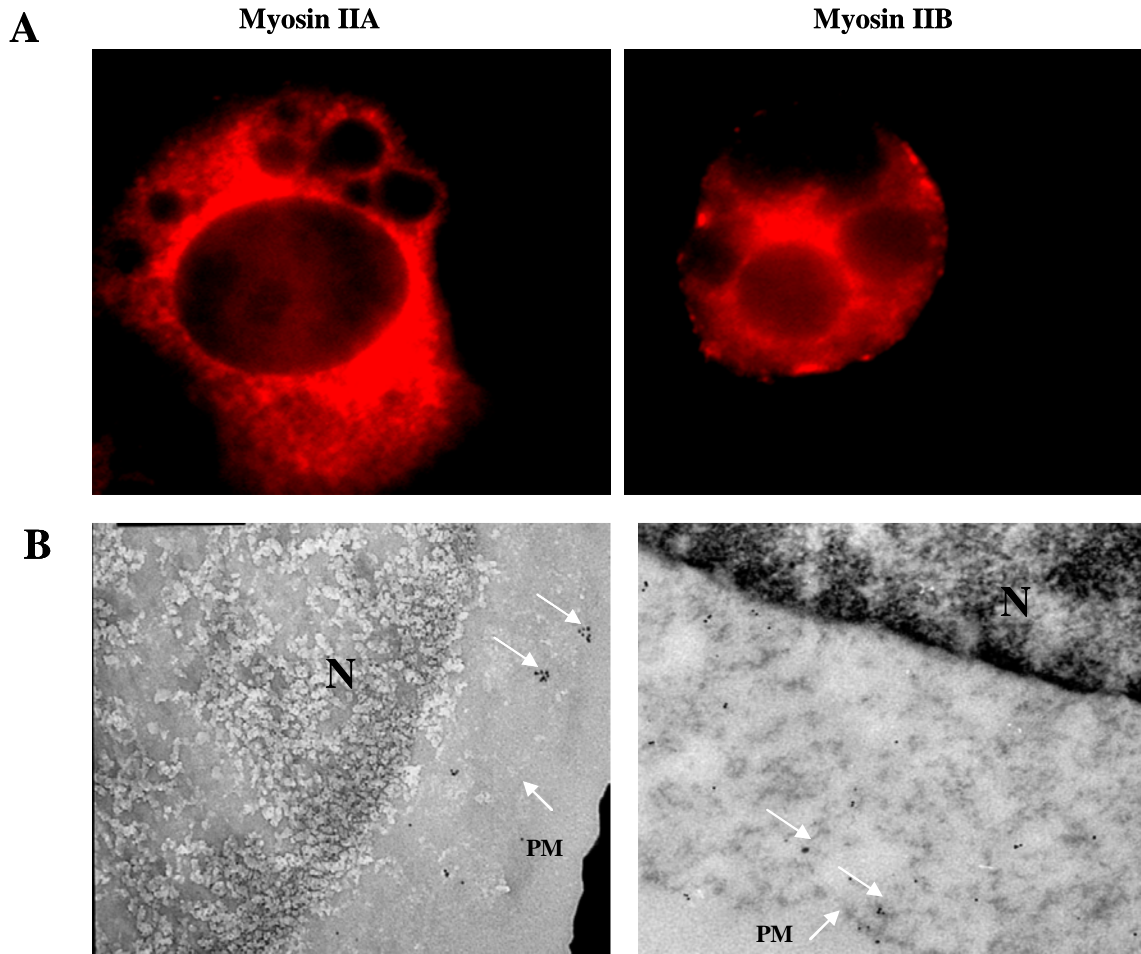

Figure 18. Localization of myosin IIA, IIB and adiponectin**.** A) Immunofluorescence staining of 3T3-L1 adipocytes with primary rabbit anti-myosin IIA (*left*) and IIB (*right*) followed by secondary anti-rabbit Alexa-fluor. B) 3T3-L1 adipocytes were fixed and processed for electron microscopy as indicated in the experimental procedures. Immunolabeling was performed with primary anti-rabbit myosin IIA (*left*) and IIB (*right*) antibody followed by donkey anti-rabbit secondary antibody 6 nM gold. Samples were visualized using a Philips transmission electron microscope. *Mag* x71,000. Figures are representative of three independent experiments.

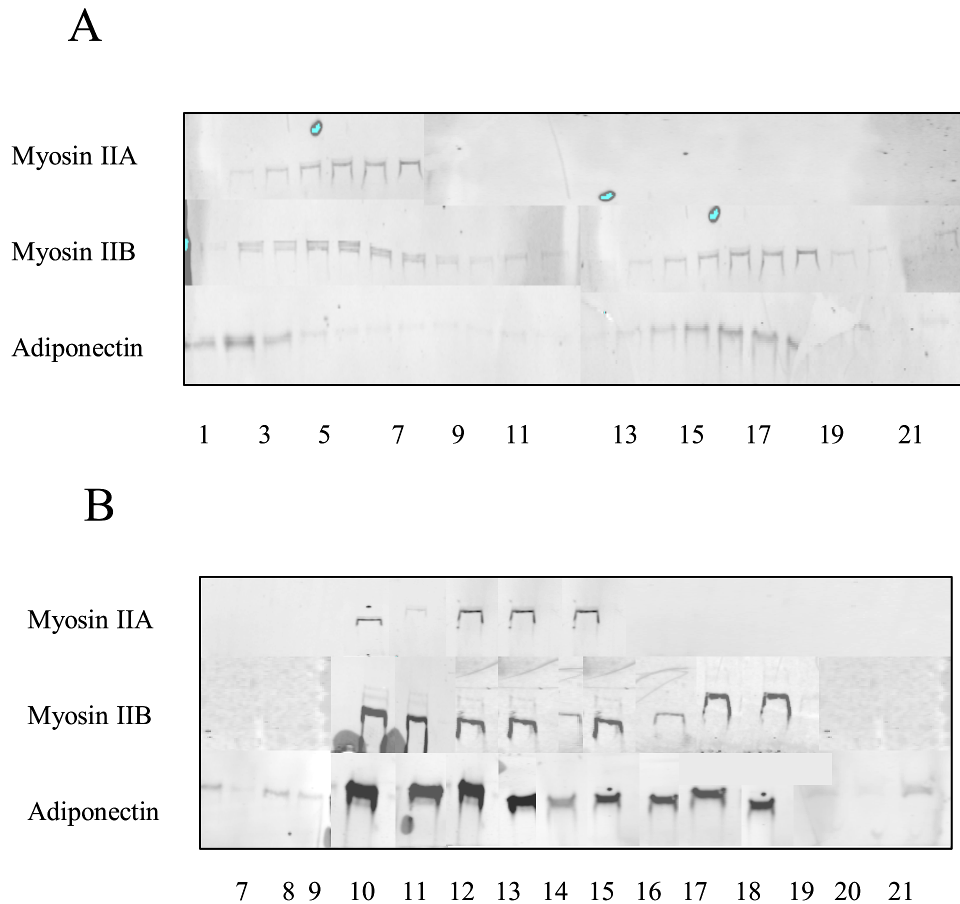

Figure 19. Adiponectin colocalizes with myosin IIA and IIB in 3T3-L1 adipocytes. A) Sucrose velocity gradient centrifugation (10-35%) of the LDM isolated from 3T3-L1 adipocytes. Fractions were collected from top to bottom (1-21). Immunoblot analysis of fractions using anti-myosin IIA, IIB and anti-adiponectin antibodies B) Equilibrium density gradient centrifugation of adiponectin-containing membranes. Fractions (1-5 and 13-19) were pooled, pelleted, loaded onto a 4 mL equilibrium density sucrose gradient (10-60%) and immunoblotted using anti-myosin IIA, IIB and anti-adiponectin antibodies.

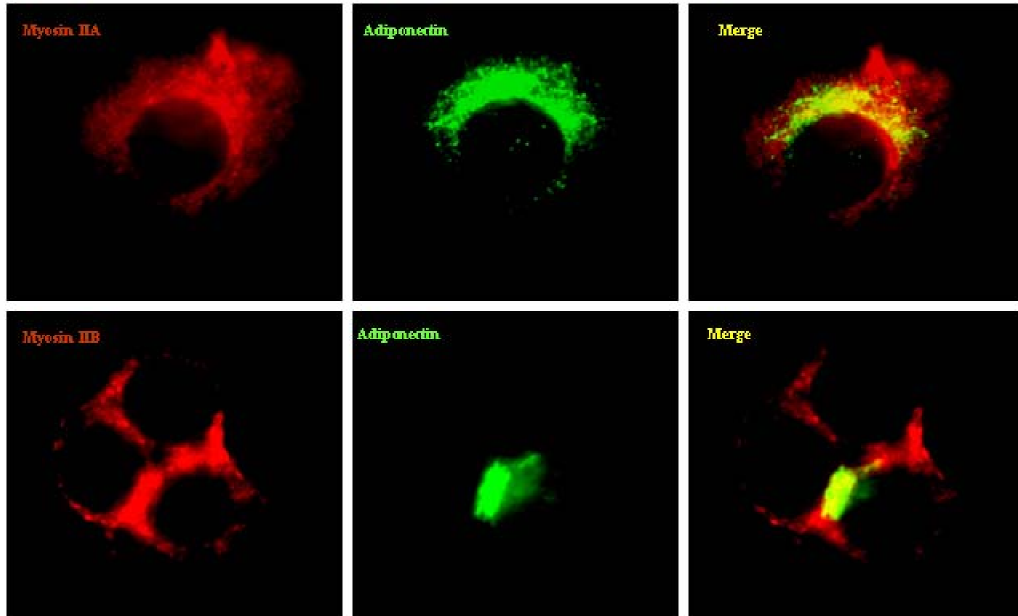

Figure 20. Adiponectin colocalizes with myosin IIA and IIB. 3T3-L1 adipocytes were fixed, permeabilized and stained for rabbit anti-myosin IIA (*left, upper*) and IIB (*left, lower*) (Alexa-fluor 595, *red*) and mouse anti-adiponectin (*center, upper and lower*) (Alexa-fluor 484, *green*). Yellow color in the merged image (*right, upper and lower*, *yellow*) indicates colocalization.

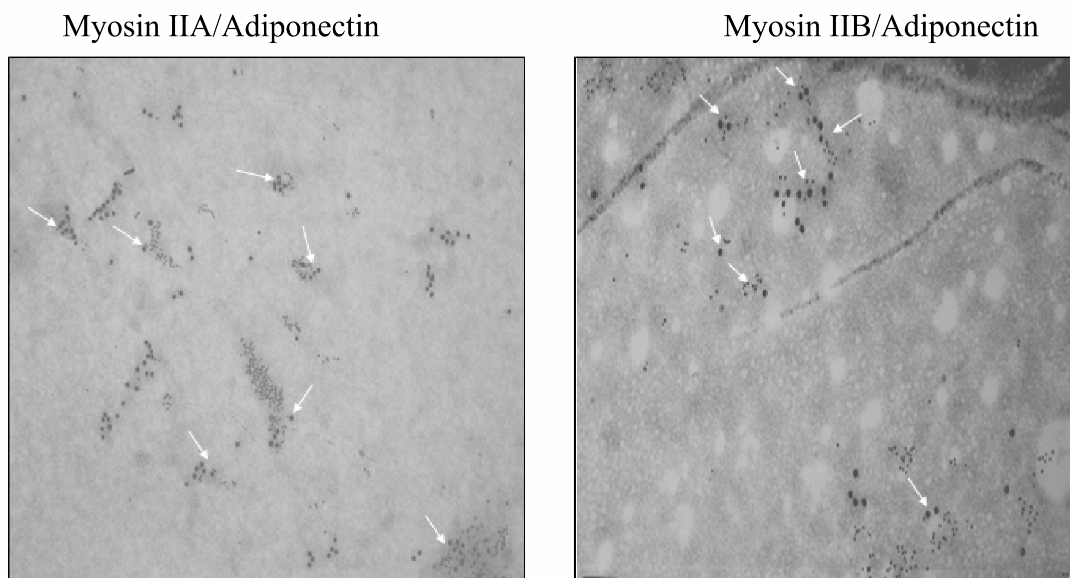

Figure 21. Adiponectin colocalizes with myosin IIA and IIB. Immunoelectron microscopy of adiponectin containing vesicles isolated by an equilibrium density gradient centrifugation were conducted as described previously by using primary mouse anti-adiponectin and rabbit anti-myosin IIA and IIB antibody followed by secondary antibodies conjugated to different size gold particles. Colocalization of myosin IIA *(left)* and IIB (*right*) (6 nM gold) with adiponectin (15 nM gold). Arrows indicate colocalization. *mag* x71,000. Figures are representative of three independent experiments.

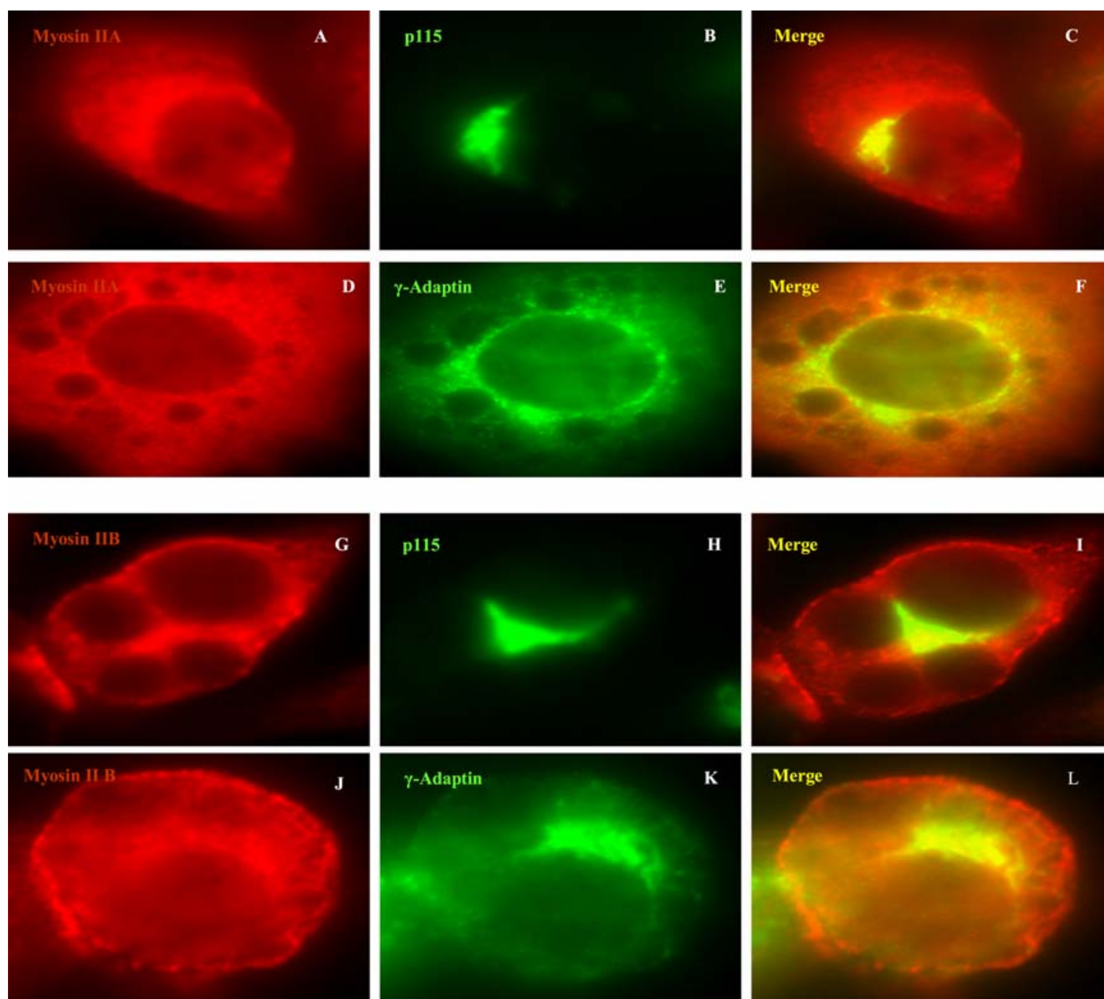

Figure 22. Myosin IIA and IIB colocalizes with the cis-Golgi marker, p115 andTGN marker, γ-adaptin. 3T3-L1 adipocytes were fixed, permeabilized and stained with anti-myosin IIA (A and D), IIB (G and J) (Alexa-fluor 594, *red*), anti-p115 (B and H) and anti-γ-adaptin (E and K) (Alexa-fluor 484, *green*). The merged images are shown in the right panel. Yellow color in the merged images (C, F, I and L) indicates colocalization.

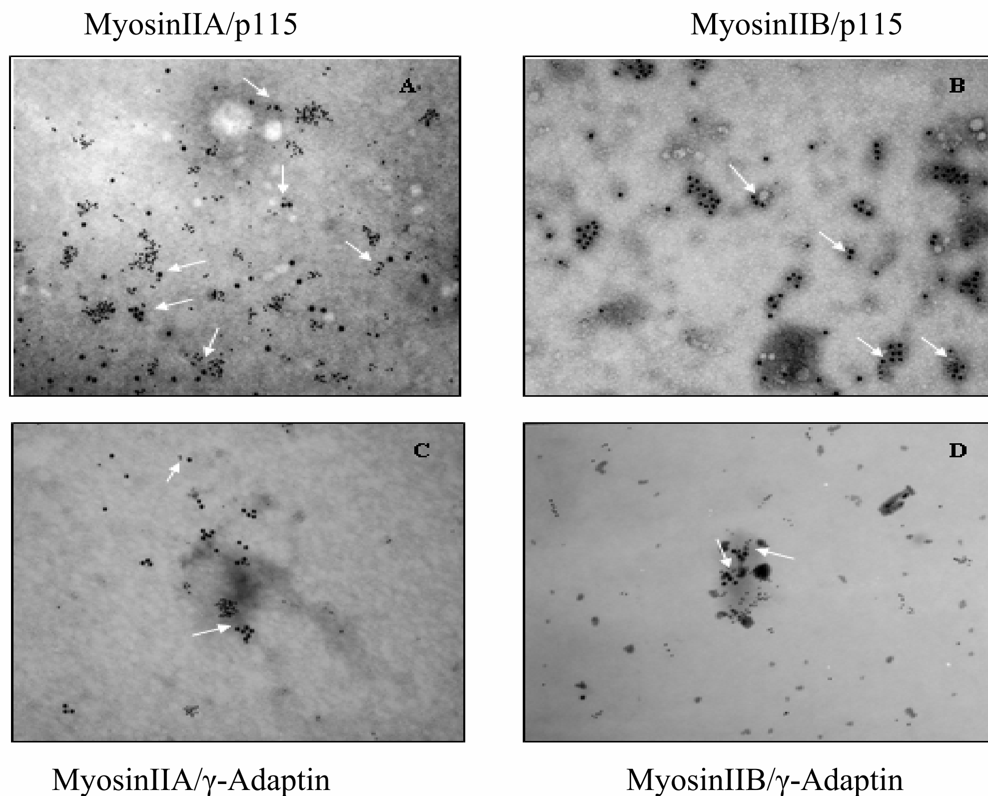

Figure 23. Myosin IIA and IIB colocalizes with the cis-Golgi marker, p115 and TGN marker, γ-adaptin. Immunogold microscopy of myosin IIA and IIB containing vesicles isolated from equilibrium density gradient centrifugation was performed using anti-myosin IIA and IIB antibodies followed by secondary antibodies conjugated to gold particles. M) Colocalization of myosin IIA (6 nM gold) and p115 (15 nM gold), N) Colocalization of myosin IIB (6 nM gold) and p115 (15 nM gold) (O) Colocalization of myosin IIA (6 nM gold) and γ-adaptin (15 nM gold) (P). Colocalization of myosin IIB (6 nM gold) and γ-adaptin (15 nM gold). *mag* x71,000. Figures are representative of three independent experiments.

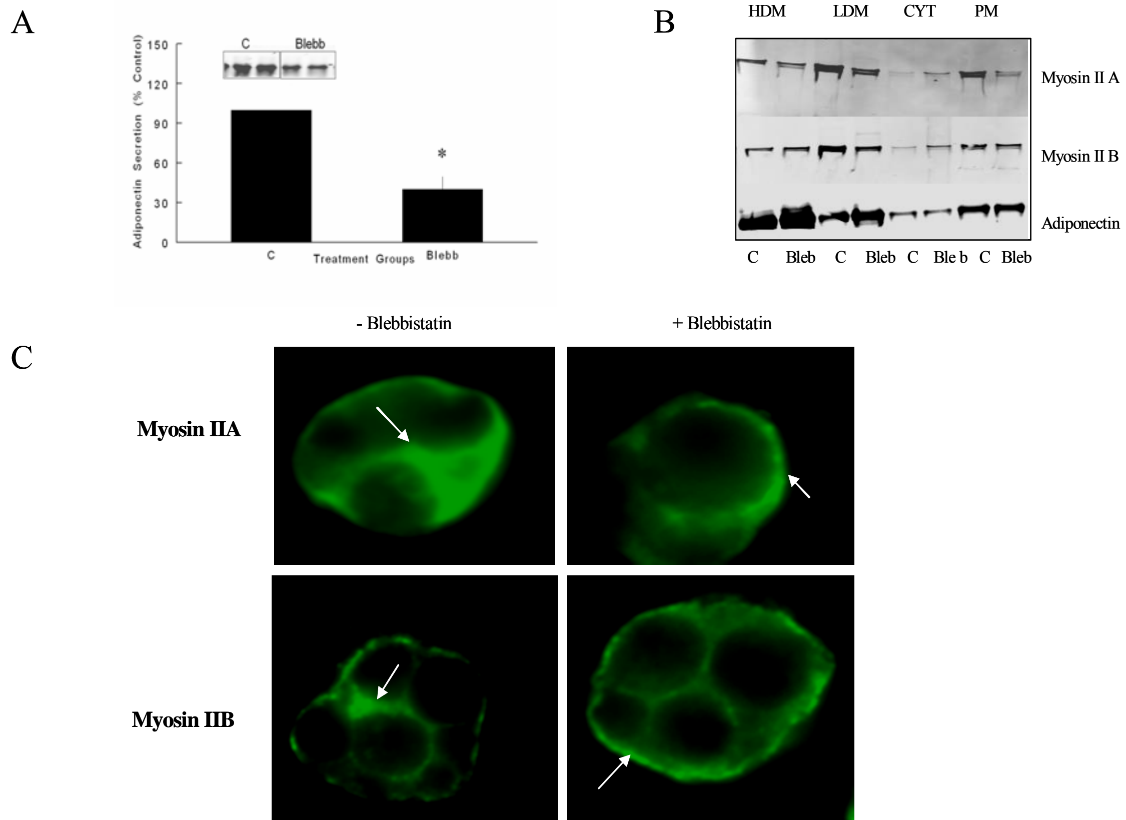

Figure 24. Blebbistatin affects myosin IIA and IIB localization and inhibits adiponectin secretion. A) 3T3-L1 adipocytes were treated with blebbistatin (Bleb) (100 μM) or vehicle for 4 hours B) Blebbistatin or vehicle treated 3T3-L1 adipocytes were subjected to differential centrifugation to yield HDM, LDM, cytosol and PM fractions. Equal amounts of protein (5 μg) were separated by SDS-PAGE and immunoblotted using anti-myosin IIA, IIB and adiponectin antibodies. C) 3T3-L1 adipocytes were treated with blebbistatin or vehicle and then fixed, permeabilized and stained with myosin IIA (*upper*) or myosin IIB (*lower*) as indicated by arrows (Alexa-flour 488 anti-rabbit, *green*). Figures are representative of three independent experiments.

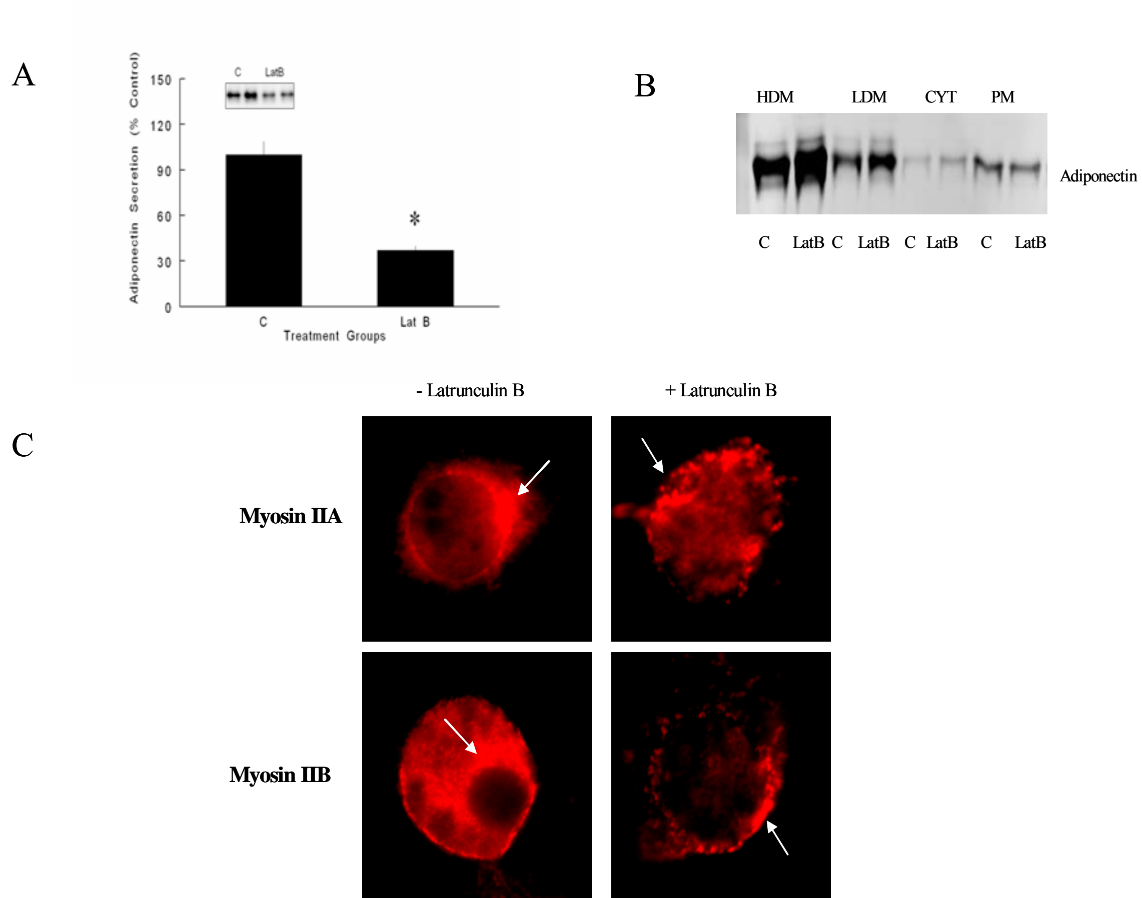

Figure 25. Latrunculin B affects myosin IIA and IIB localization and inhibits adiponectin secretion. A) 3T3-L1 adipocytes were treated with latrunculin B (Lat B) (20 μM) or vehicle for 2 hours and adiponectin secretion in the media was measured using SDS-PAGE and Western blotting. B) Latrunculin B or vehicle treated 3T3-L1 adipocytes were subjected to differential centrifugation to yield HDM, LDM, cytosol and PM fractions. Equal amounts of protein (5 μg) were separated by SDS-PAGE and immunoblotted using anti-myosin IIA, IIB and adiponectin antibodies. C) 3T3-L1 adipocytes were treated with latrunculin B or vehicle and then fixed, permeabilized and stained with myosin IIA (*upper*) and myosin IIB (*lower*) (Alexa-fluor 594 anti-rabbit, *red*). Figures are representative of three independent experiments.

CHAPTER V. CONCLUSIONS

Metabolic syndrome is a cluster of syndromes characterized by diabetes mellitus, hyperlipidemia, hypertension and visceral obesity. Insulin resistance has been considered a key factor that links visceral adiposity to adverse metabolic consequences; however, the mechanism whereby adipose tissue causes alterations in insulin sensitivity remains unclear. Adipose tissue, now recognized as an endocrine organ, secretes various adipokines that have been implicated in the development of insulin resistance. Adiponectin is one such adipokine that is produced exclusively from adipose tissue. It has insulin sensitizing, anti-inflammatory and anti-atherogenic properties and may couple the regulation of insulin sensitivity with energy metabolism. Decreased adiponectin levels are observed in patients with obesity, type 2 diabetes, hypertension and coronary heart diseases. These pathological conditions are also associated with elevated levels of ET-1, a 21 amino acid peptide, produced from endothelial cells. Increased levels of ET-1 have been associated with insulin resistance *in vivo* and *in vitro* and at the level of the adipocyte. ET-1 has been shown to induce insulin resistance via a PIP_2/actin dependent mechanism. Previous studies from our laboratory and others have suggested that ET-1 affects secretion and expression of adipokines such as leptin, resistin, and adiponectin. ET-1 has been shown to acutely stimulate and chronically inhibit adiponectin secretion from 3T3-L1 adipocytes.

In this dissertation, studies were conducted to determine the mechanism's through which chronic ET-1 administration decreases adiponectin secretion. ET-1 modulates the actin cytoskeleton in adipocytes by activating PLCβ, which stimulates the hydrolysis of PIP_2 and changes the cellular level of PIP_2. PIP_2 modulates actin dynamics in 3T3-L1 adipocytes and its loss leads to dysregulation of cortical F-actin. Studies in Chapter 3 shows that F-actin is an important regulator of adiponectin secretion and its impairement by ET-1 due leads to inhibition of basal and insulin-stimulated adiponectin secretion. Elevated levels of ET-1 has been observed in insulin resistant states and adiponectin concentrations are decreased, it is possible that one of the mechanism by which ET-1 mediates decrease in insulin sensitivity may be due to decrease in adiponectin secretion.

These studies demonstrated that adiponectin secretion is dependent on the integrity of actin cytoskeleton. Coated vesicles derived from Golgi membranes can bind to actin and are customized for differential interactions with microfilaments by the presence of selective arrays of actin-binding proteins. One of such protein is myosin II that has been shown to be involved in vesicular trafficking at the Golgi/TGN region. We next determined the role of myosin II in the intracellular trafficking and secretion of adiponectin. Our results indicates that both the isoforms of myosin II, myosin IIA and IIB, are colocalized with adiponectin in the Golgi and inhibition of myosin II activity by blebbistatin, specific myosin II inhibitor, significantly inhibits adiponectin secretion.

In summary our data not only provides evidence that ET-1, a vasoactive peptide, plays an important role in the intracellular trafficking of adiponectin, an insulin sensitizer, produced from adipocytes, but also highlights the mechanism by which adiponectin is

secreted from adipocytes. These studies also point to the fact that vascular signals such as ET-1 are capable of dysregulating its secretion, suggesting a vascular-adipocyte axis.

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