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STUDY OF ADIPOKINE SECRETION AND THEIR FUNCTIONS

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LIST OF ABBREVIATIONS

[Ca ²⁺] _i	intracellular free Ca ²⁺ concentration
3T3-L1	Mouse embryonic fibroblast- adipose like cell line
ABHD5	α/β hydrolase domain-containing protein 5
ACC	Acetyl-CoA carboxylase
ACE	Angiotensin-converting enzyme
ADD1	Adipocyte determination and differentiation factor 1
ADRP	Adipocyte differentiation-related protein
ADSC	Adipose-tissue derived stem cells
AGT	Angiotensinogen
AMP	Adenosine mono phosphate
AMPK	AMP- activated protein kinase
AngII	Angiotensin II
APJ	G protein-coupled apelin receptor
AT ₁	Angiotensin receptor type 1
AT ₂	Angiotensin receptor type 2
ATCC	American type culture collection
ATGL	Adipose triglyceride lipase
ATP	Adenosine tri phosphate
BMI	Body mass index
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer binding proteins

cAMP	Cyclic adenosine monophosphate
CBS	Calf bovine serum
cDNA	Complementary deoxyribonucleic acid
CFE	Carbon fiber microelectrode
cGMP	Cyclic guanosine monophosphate
CHO	Chinese hamster ovary cell line
CHX	Cycloheximide
CMV	Cytomegalovirus immediate early promoter
DAG	Diacylglyceride
DEX	Dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
EGFP	Enhanced green fluorescence protein
EGTA	Ethylene glycol tetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FABP4	Adipocyte fatty acid binding 4
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acid
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescence protein
GH	Growth hormone
Gi	Inhibitory GTP-binding protein

GLUT4	Glucose transporter – 4
GPCR	G-protein coupled receptor
GPDH	Glycerol-3-phosphate dehydrogenase
Gs	Stimulatory GTP-binding protein
GTP	Guanosine-5-triphosphate
HCL	Hydro chloride acid
HSL	Hormone sensitive lipase
IBMX	Isobutyl-1-methyl xanthine
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor
IL	Interleukin
IP3	Inositol triphosphate
IRS1/2	Insulin receptor substrate1/2
JAK	Janus-family tyrosine kinase
LD	Lipid droplet
LDCV	Large dense core vesicles
MAG	Monoacylglyceride
MAPK	Mitogen activated protein kinase
MGL	Monoacylglycerol lipase
NEFA	Non-esterified fatty acid
NGF	Nerve growth factor
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
PBS	Phosphate buffered saline
PC12	Pheochromocytoma cell line
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol bisphosphate

PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLL	Poly-L lysine
PPAR	Peroxisome proliferated-activated receptor
Pref-1	Pre-adipocyte factor-1
PVDF	polyvinylidene fluoride
Pyr-apelin13	pyroglutamated form of apelin-13
RAS	Renin angiotensin system
RIPA	Radio immunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
siRNA	small interference ribonucleic acid
SOCS	Suppressors of cytokine signaling
SREBP1c	Sterol-regulatory element binding protein 1c
STAT	Signal transducers and activators of transcription
SV	Synaptic vesicle
TAG	Triacyl glyceride
TBS	Tris-buffered saline
TBST	Tris-buffered saline-Tween
TIP47	Tail-interacting protein of 47 kilo-daltons
TIRFM	Total internal reflection fluorescence microscopy
TNF	Tumor necrosis factor
TZD	Thiazolidinedione

SUMMARY

Due to the recent discoveries of a growing number of cytokines secreted by adipocytes termed as Adipokines, the simple paradigm of adipocytes as merely the depot of excess energy (fat) has quickly evolved into a new notion of these cells as multi-functional endocrine cells involving in an extraordinarily broad range of physiological functions including energy metabolism. Given the essential involvements of adipokines in complex metabolic network, these secretory hormones play a pivotal role in the pathophysiology of metabolic disorders, such as obesity and diabetes mellitus. Nevertheless, the regulatory mechanism of adipokine secretion and their functions remain obscure.

We hypothesized that secretion of adipokines is highly regulated at autocrine, paracrine, and endocrine levels through cross-talks among themselves and with other metabolic factors, such as insulin, catecholamines and angiotensin II (AngII). In the current work, three types of adipokines that are critically involved in metabolic homeostasis: leptin, resistin and apelin, were focused. We studied the regulated secretion of these adipokines. We also explored the autocrine, paracrine, and endocrine functions of these adipokines, for example, regulation of leptin and resistin on catecholamine secretion from adrenal chromaffin PC12 cells, and autocrine regulation of apelin on 3T3-L1 adipocyte differentiation and lipolysis. Integrative study platform was employed, involving total internal reflection fluorescence microscopy, confocal microscopy, carbon fiber amperometry, $[Ca^{2+}]_i$ imaging photometry, molecular biology, immunoblotting and other immunoassays.

It was found that adipokines (e.g., leptin and resistin) are packed into individual secretory vesicles; vesicle trafficking and secretion of leptin and resistin are oppositely regulated by insulin/glycolytic substrates and cAMP/protein kinase A (PKA); and these adipokines

adversely influence each other on secretion and vesicle trafficking. This study highlights the autocrine interplays between leptin and resistin secreted from adipocytes. In addition, all catecholamines, which are important metabolic factors regulating energy homeostasis, inhibit vesicle trafficking and secretion of leptin and resistin through β -adrenergic receptors, while both adipokines (leptin and resistin) enhance vesicle trafficking and the release of catecholamines from PC12 cells. Our findings highlight the cross-talks between catecholamines and adipokines, and suggest the significant role of adipo-adrenal axis in energy metabolism.

Angiotensin II (AngII), which is well known for its regulation on blood vessel contraction and angiogenesis, is also produced from adipocytes. We discovered that secretion of apelin and expression of APJ receptors (apelin receptor) are differentially regulated by AT₁ and AT₂ receptors in adipocytes. Hence, by intriguing balance between the distinct AT₁ and AT₂ signaling, AngII could influence adipocyte functions via autocrine/paracrine regulations by apelin-APJ system, and control peripheral metabolic functions through endocrine regulation of released apelin. Finally, we investigated the roles of apelin on the adipocyte functions, specifically, adipogenesis and lipolysis. We provide evidences that apelin inhibits adipogenesis and lipolysis through distinct signaling pathways. This study suggests that the autocrine signaling of apelin, through interaction with APJ receptors, may serve as a novel therapeutic target for obesity and other metabolic disorders.

The studies presented herein would advance our current understanding on the regulations of adipokine secretion and the adipokine functions. Such understanding may lead to discovery of potential therapeutic targets for metabolic diseases.

CHAPTER 1 INTRODUCTION

1.1 Adipose tissue and adipocytes

Adipose tissue (fat tissue) is an innervated connective tissue which makes up of a number of different cell types, mainly adipocytes (fat cells), endothelial cells (from blood vessels), and macrophages (Fig.1). In addition to the role of adipose tissue in providing insulation and mechanical support, its main function was thought to be the major storage site for excess energy in the form of triacylglycerides (TAG) or fat. Classically, it has been known that adipocytes are able to take excess energy (glucose and fatty acids) from blood vessels, and then stored them as TAG inside the lipid droplets. Adipocytes are also able to breakdown these TAG into glycerol and free fatty acids (FFAs) again whenever necessary. FFAs can be regarded as the high energy source for the body metabolism since the metabolic process of fatty acid oxidation in the peripheral tissues (liver, muscles, and adipose tissue) can produce high energy in the form of adenosine triphosphate (ATP) (Flier, 1995; Chehab et al., 1996; Sethi and Vidal-Puig, 2007).

Most importantly, adipose tissue is increasingly regarded as the largest and active endocrine organ in the body, because of its ability to secrete a variety of hormones, growth factors, cytokines, chemokines and complement factors (Trayhurn and Beattie, 2001; Sethi and Vidal-Puig, 2007). Hormones and factors secreted from adipocytes are collectively called as the adipokines. Several studies showed that these secretory factors play pivotal roles in body metabolism and homeostasis, such as glucose and lipid metabolism, immune and inflammation functions, cardiovascular regulation and vessels formation, fat cells differentiation, fat mass regulation, etc. Given the critical roles of adipokines on metabolic pathways, these adipokines have implicated in development of various diseases and obesity related metabolic disorders,

such as insulin resistance and diabetes mellitus (Fortuno et al., 2003; Trujillo and Scherer, 2006).

Adipose tissue develops and can be found in different parts of the body, mainly in the subcutaneous layers between the muscle and the dermis. Other adipose-specific depots are found around the heart, kidneys, adrenal glands, and internal organs such as colons and small intestine. Basically, two types of the adipose tissues exist in the body, i.e., brown and white adipose tissues. Brown adipose tissue, characterized by its high content of mitochondria, is mainly for thermal regulation especially during childhood period. However, these rare brown adipose tissues, located around the neck and large blood vessels of the thorax, are mostly replaced by white adipose tissues in human adults. That is the white adipose tissues and adipocytes which stored TAG, and secrete a variety of adipokines (Sethi and Vidal-Puig, 2007; Poulos et al., 2010).

Figure 1-1 A representative model of adipose tissue and adipocytes (Tilg and Moschen, 2006)

1.2 Adipokines

The first adipokine was identified in 1994 by cloning the mouse gene, and named as leptin (from the Greek leptos for thin). Since then, numerous studies have shown the existence and importance of these circulating makers of fat reserves or adipocytes. More than 50 adipocyte-derived products or adipokines have been isolated and characterized (Lago et al., 2009). Various studies have also shown the endocrine, paracrine and autocrine functions of the adipokines on the energy metabolisms and body homeostasis. Among other adipokines, leptin, resistin, apelin and adiponectin have attracted much research attention, because they are mostly known to be significantly involved in the metabolic functions and the development of metabolic disorders (Ahima and Flier, 2000; Steppan et al., 2001a; Matsuzawa et al., 2004; Xu et al., 2011). The current knowledge on the important functions of these adipokines is summarized in Table.1.

Table.1. Major physiological functions of leptin, resistin, apelin, and adiponectin

Leptin	<p>Signals to the brain about body fat stores, and suppresses the appetite</p> <p>Regulates the energy expenditure and metabolism</p> <p>-inhibits insulin secretion from pancreas islets; stimulates catecholamines synthesis in adrenal chromaffin cells.</p> <p>-inhibits insulin-mediated glucose uptake in peripheral tissues; stimulates fatty acids oxidation in muscles</p> <p>-inhibits lipogenesis and adipogenesis; promote lipolysis in adipocytes</p> <p>Induces angiogenesis in vivo</p>
Resistin	<p>Regulates the energy expenditure and metabolism</p> <p>-reduces insulin sensitivity and enhances blood glucose level</p> <p>-impairs glucose-induced insulin releases from pancreas islets</p> <p>-modulates glucose uptake in peripheral tissues</p> <p>-Stimulates pre-adipocyte proliferation, adipogenesis and lipolysis in adipocytes</p> <p>Stimulates pro-inflammatory cytokine secretion</p> <p>Promote angiogenesis of endothelial cells</p>
Apelin	<p>Potent angiogenic factor</p> <p>Regulates vascular tone and cardiac contraction</p> <p>Regulates fluid homeostasis</p> <p>Regulates the energy expenditure and metabolism</p> <p>-modulates food intake</p> <p>-promotes insulin sensitivity and glucose uptake in peripheral tissues</p> <p>-inhibits insulin secretion from pancreas islets</p>

Adiponectin	-increase energy expenditure and fatty acid oxidation in muscles
	-suppresses body adiposity and inhibits lipolysis in adipocytes
	Regulates the energy expenditure and metabolism
	-enhances insulin sensitivity in peripheral tissues
	-increases insulin secretion from islet cells
	-increases glucose uptake and fatty acid oxidation in peripheral tissues
	-promotes adipogenesis and inhibits lipolysis in adipocytes
	Inhibitory role in inflammation and atherosclerosis
	Stimulates angiogenesis of endothelial cells

Because of their critical involvement on the major physiological functions, expression and secretion of these adipokines (leptin, resistin, apelin, adiponectin) are highly regulated. The current knowledge on the regulation of these adipokine expressions is summarized in Table.2.

Table.2. Hormonal regulation of adipocyte expressions of leptin, resistin, apelin, and adiponectin

Leptin	Insulin	stimulates leptin expression
	Glucocorticoids	stimulates <i>ob</i> gene expression
	Glucose	stimulates <i>ob</i> gene expression
	Growth hormone	inhibits <i>ob</i> gene expression
	Pro-inflammatory cytokines (TNF- α)	stimulate leptin synthesis
	Catecholamines	inhibits leptin expression
	Thiazolidinedione	inhibits <i>ob</i> gene expression
	Angiotensin II	stimulates leptin production
	Obesity	stimulates leptin expression

Resistin	Insulin	inhibits resistin gene expression
	Glucocorticoids	stimulate resistin gene expression
	Glucose	stimulates resistin gene expression
	Growth hormone	inhibits /stimulates resistin expression
	pro-inflammatory cytokines (TNF- α)	inhibits resistin gene expression
	Catecholamines	inhibits resistin gene expression
	thiazolidinedione	inhibits /stimulates resistin expression
	Obesity	inhibits resistin gene expression
Apelin	Insulin	stimulates apelin expression
	Glucocorticoids	inhibits apelin expression
	Growth hormone	stimulates apelin expression
	Pro-inflammatory cytokines (TNF- α)	stimulates apelin expression
	Obesity	stimulates apelin expression
Adiponectin	Insulin	stimulates adiponectin expression
	Glucocorticoids	inhibits adiponectin expression
	Glucose	inhibits adiponectin expression
	Growth hormone	stimulates adiponectin expression
	Pro-inflammatory cytokines (TNF- α)	inhibits adiponectin expression
	Catecholamines	inhibits adiponectin expression
	thiazolidinedione	stimulates adiponectin expression
	Obesity	inhibits adiponectin production

As shown in Table.1 and 2, various hormones and substances regulate expressions and secretions of adipokines which in turn have important roles in body physiological functions. Similarly, adipokines in turn regulate expressions and secretions of these hormones and substances which also have pivotal roles in body physiological functions. In other words, it indicates that, through the adipokines, these hormones and substances could regulate body physiological functions and homeostasis, and vice versa. For example, insulin suppresses the expression of resistin which impairs insulin sensitivity in peripheral tissues and insulin releases from pancreas islet cells. Insulin stimulates expression of adiponectin which in turn enhances insulin sensitivity and insulin secretion from pancreas islets. Imbalance of these positive/negative feedback regulations between insulin and adipokines might have adverse influences in energy metabolism.

Among these hormones and substances, catecholamines and angiotensin II are considered as the two most important hormones in body homeostasis, because of their critical involvement not only in energy metabolism, but also in other crucial physiological functions, such as blood vessels contraction and angiogenesis. For example, catecholamine is known as the potent vasoconstrictor. Angiotensin II (AngII), another potent vasoconstrictor, could also increase the angiogenesis. Interestingly, these hormones are known to have several roles in regulation on adipokine secretion and adipocyte functions.

Hence, from here onwards, I will review

- the major physiological functions of adipokines (leptin, resistin, apelin, adiponectin)
- the factors influencing expressions and secretions of these adipokines in adipocytes

In addition, two of the important hormones, catecholamines and angiotensin II, will be reviewed, because of the relevance of their involvement in energy metabolism and adipokine secretion. Finally, adipogenesis (lipogenesis) and lipolysis, two of the body physiological functions important in adipocyte-related energy metabolism, will be reviewed.

1.2.1 Leptin

Leptin, which is the best characterized prototypic adipokine, is a 167-amino acid protein (~16 kDa) encoded by the obese (*ob*) gene. Leptin was first discovered by Friedman and colleagues in 1994. Leptin is made up of four antiparallel α -helices, interconnected by two long crossover links and one short loop, and hence organized as a left-hand twisted helical-bundle. Both the nucleotide and amino acid sequences of the leptin are highly conserved among species (Radin et al., 2009). It was known that adipocyte is the major site for leptin synthesis and secretion, and hence serum leptin level is mainly contributed by adipocytes. However, there are other tissues, such as mammary gland, gastric mucosa, placenta, and liver, also synthesize to a lesser extent of leptin (Ahima and Flier, 2000). Major role of leptin is its ability to signal the body energy reserves to the central nervous system (e.g., brain), and thereby influences the food intake and metabolism (Denver et al., 2011).

1.2.2 Leptin receptor and signaling

Leptin binds to the leptin receptor (Ob-R receptor) which is encoded by the diabetes (*db*) gene. Ob-R belongs to the class I cytokine receptor family (Bjorbaek et al., 1997). Ob-Rs are produced in several spliced forms, and hence, several isoforms exist as Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re. All the Ob-Rs share an 840- amino acid of extracellular ligand-binding domain of at the amino terminal end and 34- amino acid of transmembrane domain, while differing at the carboxyl terminus. It was known that full-length isoform (Ob-Rb) is mainly considered to be involved in leptin signaling and functional receptor, however signal transductions mediated by short form has also been reported (Murakami et al., 1997; Yamashita et al., 1998).

Leptin binding of Ob-Rb, and to a lesser extent of Ob-Ra, transduces ligand-induced activation of both janus kinase family of tyrosine kinases (JAK) and signal transducers and activators of transcription (STAT) proteins. One of the consequences of the activation of STAT signaling is induction of expression of suppressors of cytokine signaling (SOCS), which

binding to Ob-Rb is known to diminish multiple leptin-induced signaling pathways, thus acting as an important negative feedback loop (Ghilardi et al., 1996). In addition to JAK-STAT signaling, leptin binding of Ob-Rb also signals via extracellular-signal regulated kinases (ERK1/2), phosphatidylinositol-3 kinase via activation of insulin receptor substrate1/2 (IRS1/2), and Akt (also known as PKB; protein kinase B) (Murakami et al., 1997; Houseknecht and Portocarrero, 1998; Sweeney, 2002). SRC homology 2 (SH2) domain-containing phosphatase 2 (SHP2) activate ERK1/2 and p38 mitogen-activated protein kinase (MAPK) signaling pathways, through the adapter protein growth factor receptor-bound protein 2 (GRB2). In addition, JAK2 can induce IRS1/2 proteins which again activate phosphatidylinositol 3-kinase (PI3K). The signaling pathway involved in leptin/Ob-Rb complex is summarized in Fig.1.2.

Figure 1-2 Signaling pathways induced by leptin receptor (Ob-Rb).(La Cava and Matarese, 2004)

Since Ob-Rb receptor mediates majority of leptin's physiological actions, lack of Ob-Rb is considered to be responsible for the *db⁻/db⁻* mouse of fat tissue mutation and obesity phenotype. Generally, Ob-Rs are highly expressed in the satiety centers of hypothalamus, while both the long and short splice variants are largely distributed in a variety of tissues and cells, including white adipocytes (Hoggard et al., 1997; Lollmann et al., 1997). Interestingly, Ob-Re, which lacks the intracellular and transmembrane domains, can be found in blood circulation, acting as a buffering system for free circulating leptin in mice (Cases et al., 2001).

1.2.3 Functions of leptin

One of the major sites of leptin action is found within the central nervous system, and through the hypothalamus, it suppresses the appetite and hence regulate the body weight homeostasis (Hoggard et al., 1997). Leptin' effects are not limited to the reduction of food intake, as leptin treatment in *ob⁻/ob⁻* mice is more effective than mere food restraint on the reduction of adiposity and the body weight (Wang et al., 1999).

Evidences of leptin effect on adipocytes have been reported. Leptin promotes lipolysis directly or indirectly by reducing insulin-mediated inhibition of adipocyte lipolysis (Zhang et al., 2009a). Leptin also directly inhibits insulin secretion in vitro and in vivo mice, while it stimulates the synthesis of catecholamines, one of the major lipolytic agents, in adrenal chromaffin cells (Fruhbeck et al., 1997), suggesting that leptin promote lipolysis through interactions with other metabolic hormones. In addition, leptin has been shown to reduce lipid accumulation by repressing gene expression of acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS), which are not under regulation of centrally mediated pathways (Chehab et al., 1996; Ducy et al., 2000). Leptin also inhibit insulin-mediated stimulation of lipogenesis. Thus, leptin has direct or indirect regulation on adipose tissue metabolism by suppressing the lipogenesis and enhancing the lipolysis (Lago et al., 2009). Recent studies have shown that, in muscle and liver, leptin increases fatty acid oxidation by activating 5-AMP-activated protein kinase (AMPK) through the direct action or indirectly through the central nervous system

(Levin et al., 1996). Taken together, leptin regulates energy and body weight homeostasis through the central appetite control as well as direct actions on the peripheral tissues.

Apart from the appetite and body weight regulation, leptin also has other diverse functions, such as reproduction (Fruhbeck, 1999), hematopoiesis (Lord et al., 1998), angiogenesis (Escobar-Morreale et al., 1997), immune functions (Blum et al., 1997), blood pressure regulation (Fruhbeck, 1999), and bone formation (Sierra-Honigsmann et al., 1998).

1.2.4 Factors influencing leptin expression in adipocytes

Leptin expression is largely regulated by the status of energy stores in fat tissues since its transcriptional expression is dependent on energy flux into adipocytes. Plasma leptin concentration correlates closely with body mass index (Tartaglia et al., 1995). Increased *ob* mRNA expression as well as serum leptin levels were observed in obese humans and other mammals (Roh et al., 2000; Niswender and Schwartz, 2003). Leptin mRNA is expressed higher in subcutaneous adipocytes than in omental adipocytes. It was known that adipocyte-size is one of the key determinants of leptin synthesis, as larger fat cells contain more leptin than smaller ones in the same individual. Transcription factors might link between leptin gene expression and adipocyte size, since the *ob* gene promoter is positively regulated via a functional binding site for CCAAT/enhancer binding protein α (C/EBP- α), while thiazolidinedione (TZD; an agonist for peroxisome proliferator-activated receptor γ , PPAR- γ) inhibits leptin expression (Frederich et al., 1995b; Cinti et al., 1997). In addition, its serum level also transiently surges following a meal and decline with fasting (Levy et al., 2000). The changes in leptin production during fasting and feeding are out of proportional to the corresponding changes in body fat or weight, suggesting that increased energy stores is not only the main factor influencing leptin expression and secretion (Cammisotto and Bukowiecki, 2004). Regulation of leptin expression by nutrition is probably mediated in part by insulin, since insulin directly stimulates leptin expression and secretion in adipocytes. In humans, leptin expression and serum level is correlated with insulin level. In contrast, leptin level is

reduced in low insulin conditions, such as in streptozotocin-induced diabetic rats while it increases after insulin treatment (Barr et al., 1997; Stepan et al., 2001a).

Leptin expression is also regulated by other metabolic and inflammatory factors as well. Glucocorticoids (Donahoo et al., 1997a) stimulate *ob* gene expression while growth hormone decreases it (Sarraf et al., 1997). Estrogen induces in vivo leptin synthesis in rats and human subjects (Trayhurn et al., 1995). Serum leptin concentration rises during the luteal phase of the estrus cycle while inversely correlated with serum male testosterone levels. Thus, effect of sex hormones on leptin expression may explain the link between sexual dimorphism and serum leptin level, in which two fold higher leptin concentration was observed in women (Hamilton et al., 1995; Grunfeld et al., 1996). Plasma levels of leptin and thyroid-stimulating hormone (TSH) are closely correlated with the body adiposity in euthyroid patient; while hypo- and hyper-thyroidism also regulate on leptin synthesis (Janik et al., 1997). Cold exposure was shown to induce sympathetic mediation as well as catecholamines suppress *ob* gene expression in adipocytes (Frederich et al., 1995a; Considine et al., 1996). In contrast, leptin synthesis in adipocytes is stimulated by pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin- 1β (IL- 1β), IL-6, and leukaemia inhibitory factor (LIF) (Lonnqvist et al., 1995; Saladin et al., 1995; Boden et al., 1996). Taken together, leptin expression in adipocytes is regulated by various factors.

1.2.5 Intracellular localization of leptin and its regulated secretion

Leptin immunostaining was first detected in the endoplasmic reticulum of adipocytes as a honeycomb pattern. After insulin treatment, leptin staining became less and was limited to a narrow band near plasma membrane of the adipocytes, however, leptin did not co-localize with Glut-4, the glucose transporter isoform found in adipocytes. By using ultrastructural localization, small cytoplasmic rim of the adipocyte revealed gold-particle labeling of leptin. Although large storage organelle for leptin could not be detected in adipocytes, small clustering of immuno gold particles could be identified in alveolate or flask-shaped structures

(40-80 nm in diameter) attached to the plasma membrane (Barr et al., 1997; Cinti et al., 1997; Holcomb et al., 2000). Cecilia roh et al reported that major pool of intracellular leptin in rat adipocytes is localized in a regulatable population of small storage vesicle like granules (Roh et al., 2000). Their highly specific and uniform distribution on sucrose gradient centrifugation also suggests that these leptin vesicles may exist in the cells as an individual secretory compartment which can be regulated (Kim et al., 2001).

Consistent with leptin localization of secretory vesicle pool, it is secreted both by constitutive and regulated pathways. After acute insulin treatment (10 min), tissue-associated leptin was lower in insulin-stimulated tissue, suggesting that leptin could be release acutely (Barr et al., 1997). Previous experiments also pointed at the role of intracellular Ca^{2+} in the process of leptin secretion. Levy et al observed that adipocytes incubated in the medium deprived of Ca^{2+} ions secreted less leptin compared to fat cells incubated with Ca^{2+} (Levy et al., 2000). This difference is pronounced in stimulated release; since glucose and insulin stimulated leptin secretion is substantially abated by Ca^{2+} deprivation (Fain et al., 2003). Taken together, these studies suggest that leptin molecules could be located in distinct secretory vesicle pool, and its secretion is related to the intracellular Ca^{2+} level.

1.3 Resistin

Resistin, one of the adipokines secreted by adipocytes, is also known as FIZZ3 (found in inflammatory zone) or ADSF (adipocyte-specific secretory factor). Resistin, a 12.5 kDa cysteine-rich protein secreted as a dimer, is made up of 108- amino acids and encoded by the gene of symbol RETN (Minn et al., 2003; Bertolani et al., 2006; Brown et al., 2007). When the cysteine (Cys-26) residue of resistin is changed to an alanine, the secreted resistin is no longer a dimeric form, but a monomeric protein (Asano et al., 2006), suggesting that this cysteine residue is important for dimerization of resistin. Each protomer of resistin molecule consists of a carboxyl-terminal disulfide-rich β -sandwich head domain and an amino-terminal α -helical tail segment. The tail-to-tail association of the α -helical segment through disulfide linkages mediates the formation of multimers (Patel et al., 2004). Protein crosslinking analysis indicated that resistin can exist as multimers by forming complex with other resistin-like molecules (RELMs) or with other resistin dimers (Fig.1.3) (Chen et al., 2002).

Figure 1-3 Representative structures of resistin and RELM molecules. [A] A protomer molecule comprise of a carboxyl-terminal globular domain and an amino-terminal helical region [B] Through disulfide bondings, three protomers forms a trimer, and two trimers form a hexamer. (Patel et al., 2004)

The amino acid homology between human and murine resistin is relatively low (~60%), while the sequences homology is larger between human, bovine, and porcine sequences (Lee et al., 2005b). In murine, resistin is almost exclusively secreted by white adipocyte tissues with lesser traces of expression found within mice brain, hypothalamus, cortex and pituitary glands (Ort et al., 2005). However, in human, resistin appears to be primarily by human macrophages although there are reports showing that resistin is, to a lesser extent, also expressed and secreted by human adipocytes (Fehmann and Heyn, 2002; Komatsu et al., 2003; Curat et al., 2006). Lower levels of resistin were also found to be present in lungs, liver, placental tissue, and pancreatic cells (Motojima, 2003; Smith et al., 2003; Lazar, 2007). The specific receptor for resistin is still unknown.

1.3.1 Functions of resistin

It is well accepted that resistin reduces insulin sensitivity and enhances blood glucose level. This has been supported by findings in which there is positive correlation between resistin expression and insulin resistance in humans (Kougias et al., 2005; Rae et al., 2007). Resistin induced insulin resistance is partly due to down regulation of insulin receptor expression in pancreatic β cells, although it has little effect on insulin secretion (Moon et al., 2003). Resistin induces the systemic insulin resistance in mice; for example, it reduces insulin-stimulated glucose uptake in rat skeletal muscle cells and mice adipose cells, while an anti-resistin antibodies produced the opposite, i.e., decrease blood glucose and improve insulin sensitivity (Lee et al., 2000). Resistin infusion in rat was shown to induce hepatic insulin resistance by increase rate of glucose production. Resistin was found to increase blood glucose levels, which is partly affecting on gluconeogenesis via inhibition of AMPK. In one report, mice lacking resistin produces low blood glucose levels after fasting, which might due to decrease hepatic glucose production, mediated by activation of AMPK and reduced expression of gluconeogenic enzymes in the liver (Delhanty et al., 2002; Banerjee et al., 2004; Li et al., 2009). Thus, all these data support the hypothesis of resistin induced insulin resistance in

rodents. However, in humans, role of resistin in insulin resistance is still far from clear. (Chiba et al., 2008)

Resistin also has effect on adipocytes too. In one report, human recombinant resistin facilitates pre-adipocyte proliferation and stimulates lipolysis in human adipocytes (Ort et al., 2005), whereas mouse recombinant resistin inhibits adipocyte differentiation (Huang et al., 2005; Bertolani et al., 2006). Consistent with these findings, acute injection of human resistin into mice cause markedly increased in serum glycerol concentration without changes in free fatty acid level. Resistin is also a pro-inflammatory cytokine, and induces up-regulation of vascular adhesion molecules. It also stimulates pro-inflammatory cytokine secretion by macrophages (Ling et al., 2001). In human macrophages, it induces lipolysis, re-esterification of triacylglycerol stores, and cholesteryl ester deposition (Maguire et al., 2009). In addition, resistin might induce atherosclerosis through the interaction with vascular endothelial cells. (Kleinz et al., 2005). Taken together, resistin play important physiological functions such as glucose and energy metabolism (Kawamata et al., 2001), however, identification of the receptor and signaling pathways will help define the more biological roles of resistin.

1.3.2 Factors influencing resistin expression and secretion in adipocytes

Several studies showed that resistin expression in adipocytes is increased in both genetic and diet-induced obesity in vivo, and decreased by a group of TZDs (Thiazolidinedione, PPAR γ -activator), an important anti-diabetic drug, including rosiglitazone, darglitazone, and troglitazone (Habata et al., 1999; Bachman et al., 2002; Morash et al., 2002). However, resistin mRNA or protein level was found decreased in different obese and insulin resistant mouse models, (Kalea and Batlle, 2010). In addition, resistin expression is suppressed in several obese rats and mice induced with diets or fed with fructose, and weight loss further suppressed resistin mRNA expression in obese rats (Fasshauer and Paschke, 2003).

In addition, it was found that resistin mRNA is reduced in *db/db* mice after rosiglitazone treatment (Banks and Farrell, 2003). However, resistin mRNA expression is up-regulated in

ob/ob mice treated with PPAR γ agonist MC-55, GW1929 and rosiglitazone (Kalea and Batlle, 2010), or in white fat tissue treated with PPAR γ agonist, NC-210060, pioglitazone, and troglitazone (Barash et al., 1996). These data suggest that resistin expression in body is tightly controlled by diets and drugs influencing insulin sensitivity. In fact, insulin and glucose are one of major factors controlling resistin expression in adipocytes. Resistin mRNA level is reduced in 3T3-L1 adipocytes treated with insulin (Kleinz and Davenport, 2005). Conversely, high glucose levels, in studies of in vitro 3T3-L1 adipocytes or in vivo hyperglycemic rats, induced resistin expression (Arner et al., 1990; Habata et al., 1999).

Several hormones also regulate resistin expression and secretion in vivo and in vitro. For example, β -adrenergic activation potently suppressed resistin expression in 3T3-L1 adipocytes, the effect is probably through Gs-coupled pathway since cholera toxin, forskolin and dibutyryl-cAMP also decrease resistin gene expression. Isoproterenol, β -adrenergic agonist, also have dose-dependent effect on resistin expression (El Messari et al., 2004). Glucocorticoid induces resistin mRNA expression in 3T3-L1 adipocytes (Habata et al., 1999). Growth hormone suppresses resistin mRNA and protein expression in 3T3-L1 adipocytes, whereas 24-hr infusion of growth hormone into spontaneous dwarf rats which lack growth hormone increase expression of resistin in adipose tissue (Masri et al., 2004). Growth hormone was also shown to induce resistin gene expression in adipose tissue of spontaneous dwarf rats (Choe et al., 2000). TNF- α (Lee et al., 2004), insulin (Kleinz and Davenport, 2005), IGF-1 (insulin like growth factor) (Sorli et al., 2006) and estrogen (Kleinz and Baxter, 2008) are a potent negative regulators of resistin mRNA expression and secretion in 3T3-L1 and mouse adipocytes. Resistin expression in the adipose tissue is also linked to prolactin and androgen level in mice (Cheng et al., 2003). Taken together, various hormones and drugs influence resistin expression in adipocytes.

1.4 Apelin

Apelin is a recently added adipokine discovered by Boucher et al in 2005 (Boucher et al., 2005). Apelin, which was first purified from bovine stomach extract in 1998 by Tatemoto and coworkers, is a novel peptide hormone and known as the only ligand of the orphaned G-protein coupled APJ receptor (Tatemoto et al., 1998). Several active forms of apelin (APJ endogenous ligand) exist in the body including apelin-36, apelin-17, apelin 13, and the pyroglutamated form of apelin-13 (Pyr-apelin13) (Fig.1.4). The apelin gene encodes a 77-amino acids prepro-protein that is cleaved into shorter amino acids forms, 36-, 17-, and 13-amino acids in size, and hence named apelin-36, apelin-17, and apelin-13. All of these apelin peptides contain the same C-terminal region, and it is thought that the bioactive region of the apelin peptides is reside in the terminal 13- amino acids fragments (Hashimoto et al., 2006). The positive charge and hydrophobic part of apelin-13 is important in interaction with APJ receptor (Fan et al., 2003). A mutation of the carboxyl terminal phenylalanine in apelin-13 (F13A) revealed a loss of apelin functions on cardiovascular system, suggesting that F13A can be considered as a competitive antagonist for APJ (Lee et al., 2005a). Moreover, the sequence of this terminal 13- amino acids fragment is highly conserved between human and animals (Fig.1.5) (Gurzu et al., 2006; Kalin et al., 2007).

Figure 1-4 Amino acid sequence of the apelin-36 [A], apelin-17 [B], Pyr-apelin-13 [C], and apelin-13 [D]. [A] ACE2 cleaves apelin-36 and apelin-13 at the position shown (Vickers et al., 2002). [C] The post-translational modification of the N-terminal glutamate of apelin-13 to pyroglutamate is revealed in dark gray color (pGlu). (Fan et al., 2003; El Messari et al., 2004). (Pitkin et al., 2010)

HUMAN	LVQPRGSRNGPGPWQGGRRKFERRQRPRLSHKGMPF
RAT	LVKPRTSRTGPGAWQGGRRKFERRQRPRLSHKGMPF
MOUSE	LVKPRTSRTGPGAWQGGRRKFERRQRPRLSHKGMPF
BOVINE	LVQPRGPRSGPGPWQGGRRKFERRQRPRLSHKGMPF ³⁶
ZEBRAFISH	PLRQNPARAGRSQRPAGWRR-RRPRPRLSHKGMPF
Xenopus laevis	LVNPKMVRNSAPQRQANRRKLIQRPRLSHKGMPF

Figure 1-5 Amino acid sequences alignment of apelin-36. Red letters indicate the residues that differ from the human sequence of apelin-36. (Pitkin et al., 2010)

Both apelin APJ receptors are widely expressed in several tissues, such as brain, heart, blood vessels, stomach, kidney and skeletal muscles (Kleinz and Davenport, 2004; Carpenne et al., 2007). Molecular forms of apelin differ among tissues. For example, apelin-36 is major component in the lung, testis, and uterus (Hashimoto et al., 2006). Apelin-13 is the predominant forms of hypothalamic and plasma apelin (De Mota et al., 2004). Major form of apelin in human cardiac tissues is Pyr-apelin 13, and it has high resistant to degradation (Dai et al., 2006). Apelin peptides can also be degraded by the enzyme, angiotensin-converting enzyme 2 (ACE2), which hydrolyze apelin with similar potency to angiotensin II (O'Shea et al., 2003). ACE2, a zinc metalloprotease, cleaves the C-terminal phenylalanine from apelin-13 and apelin-36 (Vickers et al., 2002), however, this cleavage is not important for the activation or inactivation of the peptide, since a fragment lacking this residue still retained binding and functional activities (El Messari et al., 2004).

1.4.1 APJ receptor (apelin receptor) and its signaling pathway

APJ receptor (apelin receptor) is a G-protein coupled receptor (GPCR), which displays a similar sequence homology to the angiotensin receptor type 1 (AT1 receptor), was identified in 1993 in humans 1. In CHO (Chinese hamster ovary) cells which stably transfected with human apelin receptor, apelin 36 and apelin 13 have equal ability for inhibition of adenylyl cyclase (Abdullah et al., 2008). APJ in CHO cells has been shown to be associated with α

subunit of Gq protein leading to activation of phosphorylation of ERK, which is Ras-independent way (Masri et al., 2002) . Apelin 13 is also able to activate p70S6 kinase in CHO cells through ERK dependent and PI3K dependent ways (Agarwal and Garg, 2003). Apelin-36, apelin-17 and apelin-13 are shown to produce a sharp increase in intracellular calcium concentration in neurons overexpressed with APJ (Choe et al., 2000). APJ is also expressed in human osteoblast, and found that apelin activate PI3 kinase/Akt signaling pathway (Xie et al., 2006). In rat vascular smooth muscle cells, apelin/APJ interaction activates ERK1/2 signaling through the phosphorylation of PI3K and Akt (Liu et al., 2010). Apelin signaling is also shown to block angiotensin II signaling and increase nitric oxide (NO) production in rat aortic smooth muscle cells (Chun et al., 2008). Apelin-induced glucose uptake in skeletal muscle is involved in endothelial nitric oxide synthase (NOS), AMP-activated protein kinase (AMPK), and Akt signaling pathway (Dray et al., 2008). Recently, it was found that apelin and APJ interaction in adipocytes is associated with Gi and Gq proteins, leading to reduced cAMP/PKA signaling, while promoting AMPK pathway (Yue et al., 2011).

Figure 1-6 APJ receptor signaling in rat vascular smooth muscle cells. (Liu et al., 2010)

As the one of the members of G-protein coupled receptor (GPCR), APJ receptor could be internalized. Immunocytochemical analyses revealed that APJ receptors are localized in neuronal nuclei in cerebellum and hypothalamus (Ahima et al., 1997). In CHO cells, APJ receptor was internalized through a clathrin-dependent mechanism, and apelin-17 was found to be the potent inducer for APJ internalization, and the removal of C-terminus abolished this process (al-Bagdadi, 1969). Recently, it was found that APJ receptor internalized by apelin-13 was dissociated from β -arrestin1, and then rapidly recycled to the cell surface via a Rab-4 dependent process, while internalized APJ receptor by apelin36 was sequestered intracellularly and remained associated with β -arrestin1 (Lee et al., 2010).

1.4.2 Functions of apelin

Several studies showed that apelin is a potent angiogenic factor. Apelin enhanced migration, proliferation, and capillary-like tube formation in retinal endothelial cell line RF/6A, and similarly, apelin inclusion in in vivo Matrigel plug resulted in clear capillary-like formations in the plug (Kasai et al., 2004). Inhibition of either apelin or APJ expression in frog embryo results in elimination of the formation of vascular structures, while apelin acts as a mitogenic, chemotactic and anti-apoptotic agent in mouse brain microvascular cell line bEnd3 (Cox et al., 2006). Apelin was also shown to stimulate the rate of growth and degree of vascularization in mouse tumors (Sorli et al., 2007). Up regulation of apelin-APJ pathway induces neointima formation in the mouse carotid ligation model (Kojima et al., 2010). These studies demonstrated that apelin is required for both normal and pathological conditions of vascular development.

Apelin is also important in cardiovascular system, in regulation of vascular tone, cardiac contraction and fluid homeostasis (Chandrasekaran et al., 2008). Apelin infusion in anaesthetized rat resulted in a transient drop in systolic and diastolic blood pressure, which was abolished by the pre-treatment of NOS inhibitor (Tatemoto et al., 2001). Apelin dilates venous and arterial blood vessels, and causes a mild tachycardia in conscious rodents, that is

abrogated with ganglionic blockade (Cheng et al., 2003). These suggest that apelin mediates peripheral vasodilation through NO dependent pathway. However, apelin infusion in sheep resulted in a biphasic response in mean arterial blood pressure and cardiac output, with the initial drop followed by a subsequent rise, and a return to the baseline within 15 min (Charles et al., 2006) . Apelin effect on vascular tone in vivo is confirmed by in vitro experiments. Apelin effect of arterial vasodilatation in isolated rat aortas involves L-arginine/NOS/NO pathways (Jia et al., 2007). On the other hand, in vascular smooth muscle cells, apelin activates myosin light chain, which could initiate smooth muscle contraction (Hashimoto et al., 2006) . Similarly, in human saphenous veins, apelin causes venous vasoconstriction in the absence of a functional endothelium (Katugampola et al., 2002). Apelin also interacts with AngII in order to regulate on vascular tone. Apelin also interact with AngII in order to regulate on vascular tone. Apelin abolishes the vasoconstriction effect of AngII, which is also a NO dependent mechanism (Gurzu et al., 2006). In AJP knockout mice, there is increased vasopressor response to AngII (Ishida et al., 2004).

Apelin is a powerful inotropic factor in vivo too. Chronic apelin infusion causes increased cardiac output in conscious mice. And this inotropic effect is not through NO synthase pathway, adrenergic signaling, or cardiac innervation; but it is thought to be through increase intracellular concentration (Ashley et al., 2005). This increase in cardiac muscle contraction is seemed to be more powerful in failing heart (Dai et al., 2006). Apelin is also involved in regulation of fluid homeostasis through interaction with antidiuretic hormone (ADH) (Reaux et al., 2001).

As a member of adipokines, apelin is also important in energy metabolism. Evidences showed that central apelin has a role in regulation of food intake and feeding behavior (Rayalam et al., 2008). Apelin-13 injection in lateral cerebral ventricles decreased 24 food intakes in both fed and fasted rats (Sunter et al., 2003), while apelin-12 injection stimulated food intake during the day and reduced intake at night (O'Shea et al., 2003). In addition, apelin-13 injection into third cerebral ventricles was found little or no effect on food intake (Taheri et al., 2002), while chronic apelin-13 injection resulted in an early increase in food

intake and increase weight gain in rats (Valle et al., 2008). Higuchi et al found that intra-peritoneal injection of apelin had little effect on food intake, while body adiposity and serum triglyceride level were decreased in apelin-treated mice. Apelin induced expression of uncoupling protein -1 (UCP1) in brown adipose tissue, and hence increase energy expenditure could be one of possible factors for apelin suppressed body adiposity (Higuchi et al., 2007). Yue et al also found that body adiposity and serum free fatty acid level was increased in apelin-null mice, which could be reversed in apelin treatment (Yue et al., 2011).

In addition, many studies showed that apelin has pivotal role in glucose metabolism. First, apelin inhibits glucose induced insulin secretion in vivo mice as well as in vitro isolated islets of Langerhans (Sorhede Winzell et al., 2005; Guo et al., 2009). Intravenous apelin injection stimulates glucose utilization in normal and obese mice, which is mainly through increased glucose uptake in skeletal muscles and adipose tissues (Dray et al., 2008). Apelin administration in apelin signaling null mice (APKO) and *db/db* mice resulted in improved insulin signaling, and suggesting that apelin is necessary for maintenance for insulin sensitivity in vivo (Yue et al., 2010). Similarly, apelin was able to increase glucose uptake in insulin-resistant 3T3-L1 adipocytes 24 pre-treated with $\text{TNF}\alpha$, which is through PI3K/Akt pathway. Immunocytochemistry staining revealed that apelin enhances the translocation of glucose transporter 4 (Glut-4) to the plasma membrane (Zhu et al., 2011). Recently, autocrine/paracrine role of apelin on adipocyte lipid metabolism was revealed, in which apelin was shown to suppresses isoproterenol induced lipolysis in both isolated adipocytes and 3T3-L1 differentiated adipocytes (Yue et al., 2011). These data highlight that apelin has important role in glucose and lipid metabolisms; however, autocrine/paracrine effects of apelin on adipocytes are still largely unknown.

1.4.3 Factors influencing apelin expression and secretion in adipocytes

Boucher et al has shown that apelin was not only expressed but also secreted by adipocytes; and since then, many studies have found that several factors were regulating on apelin

expression and secretion from adipocytes. There is a relationship between apelin and insulin both in vitro and in vivo, while plasma apelin level is increased in obese with hyperinsulinemic mice. Apelin expression in adipocytes is increased by insulin treatment, while apelin mRNA is reduced in adipocytes collected from streptozotocin-treated mice (absence of insulin). Fasting and re-feeding modulate the pattern of apelin expression in adipocytes, which correlates with the pattern of plasma insulin levels (Boucher et al., 2005). $\text{TNF}\alpha$, a cytokine elevated in conditions like obesity and insulin resistance, also induced apelin expression in adipocytes (Daviaud et al., 2006). In addition, basal and insulin induced apelin expression and secretion is increased in adipocytes treated with eicosapentaenic acid (EPA), a omega-3 family of polyunsaturated fatty acid (PUFA) (Lorente-Cebrian et al.). Overexpression of PGC1 α (peroxisome proliferator-activated receptor γ (PPAR γ) coactivator-1 α) which is important in energy homeostasis in oxidative states, also stimulate apelin secretion and expression in human adipocytes (Mazzucotelli et al., 2008). Plasma apelin level is increased in obese subjects (Castan-Laurell et al., 2011). Apelin and APJ receptor expression in human adipose tissue is increased with obesity, while this could be reversed upon diet-induced weight loss (Castan-Laurell et al., 2008). These data suggest that apelin expression and secretion in adipocytes are modulated by a wide range of metabolic relevant factors, however further studies are needed to better define the regulation of apelin/APJ system in adipocytes.

1.5 Adiponectin

Adiponectin, which was first discovered in mid-1990s by four independent groups and namely apM1, Acrp30, GBP28, AdipoQ, is produced almost exclusively from mature adipocytes. Adiponectin is a 244- amino acid protein. It contains four domains: a signal sequence, a variable domain, a collagen-like domain, and a globular C-terminal region (Kadowaki and Yamauchi, 2005). Adiponectin, a 28 kDa in size, is secreted by adipocytes as homotrimers, and in circulation, it may form trimers, hexamers, or high molecular weight multimers (HMW) (Fig.1.7). Low molecular weight (LMW) trimmers are produced through association of globular and collagenous domains. Higher order interaction of adiponectin depends on disulfide bond formation mediated by cysteine-39 (Cys-39) (Pajvani et al., 2003). Interestingly, the globular domain of adiponectin, which was generated by proteolytic cleavage of adiponectin by leukocyte elastase secreted from activated monocytes, may also circulate in the blood circulation (Waki et al., 2005). Currently, based on clinical observations, the HMW forms is thought to be the most biologically active form, and best associated with adiponectin's action of increased insulin sensitivity (Radin et al., 2009).

Figure 1-7 Adiponectin structure. **[A]** Signal sequence, variable domain in N-terminus and the globular had domain in the C-terminus of adiponectin. **[B]** Adiponectin monomers could associate into trimmers, low molecular weight (LMW) and high molecular weight (HMW) through the disulfide bonds. All of these could be secreted from the adipocyte. (Frizzell et al., 2009).

1.5.1 Adiponectin receptors and signaling pathways

Adiponectin exerts its biological activities by binding to two structurally and functionally distinct receptors, AdipoR1 and AdipoR2 (Fig.1.8). AdipoR1 is mainly expressed in skeletal muscle, and it has higher affinity for the globular and trimeric forms. AdipoR2 is primarily expressed in liver and it has more affinity for the hexameric and HMW forms. Both receptors, the integral membrane proteins which composed of an internal N-terminus and external C-terminus, activate signaling molecules such as peroxisome proliferator-activated receptors- α (PPAR α), AMP-activated protein kinase (AMPK) and mitogen-activated protein kinase (p38 MAPK). In skeletal muscle, adiponectin activates AMPK and PPAR α , and hence stimulates phosphorylation of acetyl CoA carboxylase (ACC) and fatty acid oxidation, and induces glucose uptake in muscles. In liver, activation of AMPK by adiponectin reduces gluconeogenesis, and increases phosphorylation of ACC and fatty acid oxidation. Taken together, adiponectin's dual effects on liver and muscle contribute to overall increased in insulin sensitivity, while reducing blood glucose and tissue TAG levels (Fig.1.9) (Yamauchi et al., 2003).

Figure 1-8 Structure of adiponectin receptors, AdipoR1 and AdipoR2. AdipoR2 is 66.7% amino acid identity with AdipoR1. Both contain structurally and topologically distinct seven-transmembrane domains. (Kadowaki et al., 2006)

Figure 1-9 Signal transduction of adiponectin receptors in liver and muscle. (Kadowaki et al., 2006)

Adiponectin receptor expression has also been studied in human adipose tissue. In both adipose tissue and isolated adipocytes, AdipoR1 mRNA expression is ~10 times higher than that of adipoR2; however, an adipoR1 mRNA level is lower in adipose tissue extracted from obese subjects. AdipoR1 expression in adipose tissue is negatively correlated with body mass index (BMI), while weight loss produced increased AdipoR1 expression in adipose tissue (Rasmussen et al., 2006). Since plasma adiponectin level is also significantly lower in obese subjects, lower levels of adiponectin receptors in obesity might aggravate the already reduced metabolic effects of adiponectin in obese state.

1.5.2 Functions of adiponectin

The role of adiponectin in body has been addressed by many studies. Probably, the most important functions of adiponectin include its enhancement on insulin sensitivity, its inhibitory effects on proliferation, inflammation, and the development of atherosclerosis (Hopkins et al., 2007). In peripheral tissues, adiponectin-induced AMPK activation increase glucose uptake by increasing the translocation of GLUT4 to the cell surface; increase glycolysis via phosphorylation of phosphofructokinase; and increase fatty acid oxidation by phosphorylation of acetyl CoA carboxylase (ACC) (Kadowaki et al., 2008). In addition, full-length adiponectin enhances the insulin-induced reduction of glucose output in liver cells; and this effect is likely through reduced expression of gluconeogenic enzymes in liver (Berg et al., 2001; Yamauchi et al., 2002). Studies also showed impaired insulin sensitivity in adiponectin knockout mice (Kubota et al., 2002; Maeda et al., 2002). Plasma adiponectin levels are lower in diabetic patients; and according to several clinical studies, it was well accepted that individual with relatively high adiponectin level is less likely to develop type 2 diabetes mellitus, while low plasma level is associated with future development of diabetes mellitus (Hotta et al., 2001; Snehathatha et al., 2003; Spranger et al., 2004).

Roles of adiponectin in cardiovascular function have been reported. Adiponectin protect heart from the ischemia or reperfusion injury by reducing oxidative stress mediated by nitride oxide (NO) and it was known that high adiponectin level is correlated with reduced incidence of myocardial infarction (Pischon et al., 2004; Tao et al., 2007);. Cardiac events are more prone in patients with lower adiponectin level. Ischemia/reperfusion in adiponectin-null mice resulted in increased myocardial apoptosis and infarct size, while infusion of adiponectin reversed these conditions. Adiponectin suppressed the apoptosis in cardiac muscle and fibroblast is also dependent on AMPK activity (Shibata et al., 2005). In addition, adiponectin is also considered as the novel hormonal vasodilator. Adiponectin promotes vasorelaxation through the increased vascular expression of endothelial nitric oxide synthase and prostacyclin synthase, which is likely through the stimulation of AMPK activity (Ouchi et al., 2003).

Recent study has demonstrated that adiponectin has ability to relax the isolated aortic and mesenteric arteries through the opening of K^+ channels, which is independent of the presence of the endothelium (Fesus et al., 2007).

Adiponectin has inhibitory role in inflammation and atherosclerosis. These functions are likely through its abilities to suppress $TNF\alpha$ production in macrophage and to prevent myelomonocytic progenitor cell growth (Yokota et al., 2000). Adiponectin treatment reduced the expression of adhesion molecules: intracellular adhesion molecules-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1) and E-selectin, and hence subsequently reduce the adherence of monocyte (Ouchi et al., 1999). Thus, these prevent monocyte movements into subendothelial areas, and hence prevent the development of atherosclerotic plaques. Adiponectin also suppress the expression of the scavenger receptor class A-1 (SR-A) in macrophage, and hence reduced uptake of oxidized LDL and inhibition of foam cell formation (Ouchi et al., 2001). In addition, adiponectin suppresses the proliferation and migration of vascular smooth muscle cells, through competitive binding of platelet-derived growth factor-BB (Arita et al., 2002). It has been shown that adiponectin suppressed the $TNF\alpha$ induced secretion and expression of interleukin8 (IL8), a pro-inflammatory cytokine that promote atherogenesis, in human aortic endothelial cells, confirming the role of adiponectin on inhibition of atherosclerosis (Kobashi et al., 2005).

Growing evidences suggest that adiponectin is also important in regulation of lipid metabolism. Plasma adiponectin level is positively correlated with plasma HDL level, and negatively correlated with plasma triglyceride levels (Cnop et al., 2003; Tschritter et al., 2003). Adiponectin also has paracrine or autocrine effects on adipocytes too. It increases adipogenesis with the increased expression of adipogenic related transcriptional factors, such as $C/EBP\alpha$, $PPAR\alpha$, and adipocyte determination and differentiation factor 1/sterol-regulatory element binding protein 1c (ADD1/SREBP1c). Adipocytes overexpressed with adiponectin accumulated more and bigger lipid droplets compared with control cells. In addition, it increase insulin stimulated glucose uptake in adipocytes through increased GLUT4 gene expression and recruitment to the plasma membrane (Fu et al., 2005).

1.5.3 Factors influencing adiponectin expression and secretion in adipocytes

Plasma adiponectin level and its expression in fat are reduced in obese and insulin resistant stages (Hotta et al., 2001; Weyer et al., 2001) while adiponectin expression is stimulated with increased insulin sensitivity and weight loss (Yang et al., 2001). TZDs (Thiazolidinedione) induced adiponectin mRNA and its secretion level in vitro and in vivo conditions (Fasshauer et al., 2004). Long-term rosiglitazone treatment in diabetes patients increases plasma adiponectin level (Yang et al., 2002a). In addition, hormones that reduced insulin resistance down-regulate the adiponectin expression; for example, β -adrenergic agonists inhibits adiponectin expression in differentiated 3T3-L1 adipocytes, which is mediated through Gs protein coupled pathways (Fasshauer et al., 2001a). Insulin also has direct effect on adiponectin expression level. Insulin stimulates adiponectin mRNA expression in human and mouse white adipocytes. (Halleux et al., 2001) However, long-term insulin treatment reduces adiponectin expression in 3T3-L1 adipocytes in vitro (Fasshauer et al., 2002). Similarly, plasma fasting insulin level is negatively correlated with plasma adiponectin level in vivo (Bluher et al., 2002; Yu et al., 2002).

A number of hormones and factors also control adiponectin expression and secretion from adipocytes. In fact, many factors have inhibitory effect on adiponectin expression including catecholamine, glucocorticoids, cytokines (IL-6 and TNF- α), prolactin, growth hormone, and androgens. Glucocorticoids suppressed adiponectin mRNA expression in human and rodents adipocytes (Fasshauer et al., 2002) Increased local glucocorticoid level by overexpression of 12- β -hydroxy steroid dehydrogenase in fat tissue also decreased adiponectin expression in vivo (Masuzaki et al., 2001). Testosterone therapy decrease plasma adiponectin level suggesting the sex difference in plasma adiponectin levels, consistent with the studies found that females have higher total circulating adiponectin levels compared with males (Nishizawa et al., 2002) Increased production of pro-inflammatory cytokines such as TNF- α and IL-6 as well as other reactive oxygen species within the enlarging adipose tissue also inhibits adiponectin gene expression (Kappes and Loffler, 2000; Fasshauer et al., 2003) In addition,

leptin, retinoic acid as well as IGF-1 may also play a role in regulating adiponectin gene expression and secretion (Zhang et al., 2002). Various dietary supplements such as fish oil, linoleic acid, soy protein, etc, have been shown increasing the adiponectin levels in various rodent models (Radin et al., 2009).

1.6 Catecholamines

Catecholamines, also known as the fight-or-flight hormones, are a group of molecules that contain a catechol or 1,2-dihydroxybenzene group, which derived from the amino acid tyrosine (Fig.1.10). Most important catecholamines in the body are epinephrine (adrenaline), norepinephrine (noradrenaline), and dopamine (Eisenhofer et al., 2004). Adrenaline was first discovered in 1900 by Takamine and his coworkers (Bennett, 2003). Dopamine was discovered in 1952 by Carlsson and his coworkers, and named because of its precursor 3,4-dihydroxyphenylalanine (L-DOPA). Catecholamines are mainly secreted from nerve endings of the sympathetic neurons, and hence involved in neurotransmission and subsequent activation of sympathetic nervous system. Major sympathetic effects are to increase heart rate, breathing rate, blood pressure, and mental alertness (Goldstein, 2003).

Figure 1-10 Biosynthesis of catecholamines from tyrosine.

Catecholamines are also secreted from chromaffin cells of the adrenal medulla located above the kidney. Medullary chromaffin cells are modified post-synaptic sympathetic neuroendocrine cells that receive sympathetic input. Released from these chromaffin cells, catecholamines (80% epinephrine and 20% norepinephrine) enter into systemic circulation, and subsequently produce the systemic effects on various organs and tissues. In body, epinephrine and norepinephrine are the primary endogenous ligands for adrenergic receptors: β_1 , β_2 , β_3 and α_2 adrenergic receptors, while dopamine is the ligand for dopamine receptors: D1, D2, D3, D4 and D5 receptors. Although their structures are similar, their distributions in the central and peripheral nervous system and in the endocrine system, as well as their functions in the body are versatile. Given that catecholamines occupy key positions in the regulation of physiologic functions (especially energy metabolism) as well as the development of neurological, psychiatric, endocrine and cardiovascular disorders (Axelsson, 1971), these hormones continue to receive considerable research attention.

1.6.1 Role of catecholamines in adipocyte

Probably the most important function of catecholamine is its role of regulation in lipolysis, breakdown of triglycerides or fat into fatty acids and glycerol. Many have reviewed in detail about the mechanism by which catecholamine regulates lipolysis in human fat cells which express all the adrenergic receptors for binding of catecholamines. Specifically, catecholamines bind to β_1 , β_2 , β_3 adrenergic receptors which are coupled to stimulatory GTP sensitive proteins and hence activate the membrane bound enzyme adenylyl cyclase, and subsequently increase cyclic AMP (cAMP) and activate protein kinase A (PKA). PKA phosphorylates hormone sensitive lipase (HSL) which catalyzes the breakdown of triglycerides (lipolysis). Catecholamines can also bind to α_2 adrenergic receptor producing the opposite effects on lipolysis. α_2 adrenergic receptor is coupled to inhibitory GTP sensitive proteins, and activation of α_2 receptor inhibit adenylyl cyclase, cAMP formation, PKA activation, HSL phosphorylation and finally the lipolysis (Arner, 1999).

1.6.2 Cross-talks between catecholamines and adipokines

Several evidences have shown that catecholamines regulate the production of adipokines which in turn regulate on catecholamines synthesis and secretion. Catecholamines and synthetic β_3 adrenergic agonists rapidly inhibit the leptin mRNA expression in mouse adipocytes. Inhibitory effect of catecholamines could be blocked by β adrenergic antagonist propranolol, suggesting that all the β_1 , β_2 and β_3 receptor are involved in the regulation of leptin expression (Trayhurn et al., 1995; Trayhurn et al., 1996). On the other hand, Leptin inhibits the release of catecholamines (norepinephrine and dopamine) from the hypothalamic neuronal endings, suggesting that both catecholamines and leptin regulate expression and release of each other (Brunetti et al., 1999).

Isoproterenol, β adrenergic receptor agonist, inhibits resistin gene expression through a Gs protein coupled pathway in 3T3-L1 adipocytes (Fasshauer et al., 2001b), while resistin inhibits dopamine and norepinephrine release in hypothalamus (Brunetti et al., 2004). In addition, norepinephrine and β_3 adrenergic agonists stimulated IL-6 gene expression and protein secretion in primary cultured murine adipocytes (Burysek and Houstek, 1997), while IL-6 increase catecholamine secretion in rat carotid body glomus cells (Fan et al., 2009). Furthermore, β adrenergic agonists up regulate $\text{TNF}\alpha$ and down regulate the adiponectin expression in adipocytes (Fu et al., 2007b), while $\text{TNF}\alpha$ modulates the inactivation of catecholamine secretion from culture sympathetic neurons (Soliven and Albert, 1992). Based on these observations, it is obvious that catecholamines and adipokines influence each other on expression and secretion.

1.7 Angiotensin II

Angiotensin II (AngII) is a peptide hormone known to play a major role in regulating blood pressure, fluid and electrolyte balance, and cardiovascular homeostasis. It is the part of the renin-angiotensin system (RAS) (Fig.1.11), which is considered as the circulating endocrine system regulating a broad range of physiological functions. Systemic or circulating RAS begins with the liver-production of angiotensinogen (AGT), precursor of all kinds of angiotensin peptides, which is activated or cleaved by a hormone, renin, released from the juxtaglomerular cells of the kidney. Cleaving of AGT then produces the inactive decapeptide angiotensin I (AngI) which is then converted to the active octapeptide AngII in pulmonary circulation by the action of an enzyme, angiotensin-converting enzyme (ACE) (de Kloet et al., 2010).

Figure 1-11 Renin-angiotensin system and its components (Hunyady and Catt, 2006).

In addition to this classical RAS, several studies indicate that many tissues, including the brain, kidney, heart, blood vessels, and adipose tissue, express the components of the RAS, such as AGT, renin, and ACE. And hence these tissues are capable of producing AngII (Engeli et al., 2000; Chu and Leung, 2009; de Kloet et al., 2010). Moreover, there are alternative pathways which also could generate AngII in tissues through the non-renin and non-ACE pathways, by using other enzymes including chymase, cathepsin G, tissue plasminogen activator (tPA), chymostatin-sensitive AngII-generating enzyme (CAGE), and tonin (Urata et al., 1995; Urata et al., 1996). Furthermore, AngII can also convert into several other forms of angiotensin peptides (e.g., Ang(1-7), AngIV) which mediating different physiological effects. For example, Ang(1-7) is believed to play a major role in counterbalancing the effects of AngII (Fyhrquist and Saijonmaa, 2008). Therefore, with growing complexity of RAS system, angiotensin peptides become more important in regulation of a wide range of body homeostatic functions.

1.7.1 Angiotensin receptors

AngII mediates its function mainly through two angiotensin receptor subtypes, designed angiotensin receptor type-1 (AT₁) and angiotensin receptor type-2(AT₂) (Griendling et al., 1996; Dinh et al., 2001). Angiotensin receptors belong to the group of seven-transmembrane spanning G-protein coupled receptors. The amino sequence of these receptors are highly conserved among species, however, sequence homology of AT₁ and AT₂ receptors share only about 34%, and also have distinct signaling transduction pathways. Virtually all the known physiological functions of AngII are thought to be mediated by AT₁ receptor; however, there is emerging role of AT₂ receptor in regulating body homeostasis (Burnier and Brunner, 2000; Dinh et al., 2001; Steckelings et al., 2010).

1.7.2 AT₁ receptor and its signaling pathways

AT₁ receptor has a molecular weight of 41 kDa, and consists of 359- amino acids. There are two types of AT₁ isoform in rodents, AT_{1A} and AT_{1B}, and they share 94% sequence similarity (Bottari et al., 1993; Miura et al., 2011). However, in human, there is only a unique gene coding for AT₁ receptor. The transmembrane domain and the extracellular loop of AT₁ receptor are important for AngII binding; however, there are different binding sites for other AT₁ receptor antagonists which could only interact with the transmembrane domain. Several studies have demonstrated that AT₁ receptor is expressed in a variety of tissues and cells, including the brain, adrenals, heart, vasculature, kidney, and adipose tissues (Griendling et al., 1996; Dinh et al., 2001) . It was known that, in human pre-adipocytes and adipocytes, predominant form of angiotensin receptor is the AT₁ receptor subtype; however, both the AT₁ and AT₂ subtypes are abundantly expressed in rodent's adipocytes (Engeli et al., 1999; Schling et al., 1999).

Many studies have reviewed the detailed signaling pathways involved in AT₁ receptor activated by AngII (Fig.1.12) (Dinh et al., 2001; Bruno et al., 2005; Hunyady and Catt, 2006). Upon activation of AT₁ coupled Gq/11 protein, activation of phospholipaseC- β occurs, which subsequently catalyzes on phosphatidylinositol 4,5-biphosphate (PIP₂) into two secondary messengers, inositol triphosphate (IP₃) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), while IP₃ stimulates the release of Ca²⁺ from intracellular stores. In addition, in some case, AT₁ receptor activates the phospholipase A₂ and D, which stimulate the release of arachidonic acids, and subsequently generate the prostaglandins. AngII mediated AT₁ receptor also linked to Gi/o protein which inhibit adenylate cyclase in several tissues, and hence attenuate the production of cAMP. In some tissues, especially in blood vessels, AT₁ receptor is involved in opening of Ca²⁺ channels and hence influx of extracellular Ca²⁺ into the cells. Increased Ca²⁺ level and PKC activity, and decreased cAMP level are linked to the AngII action of vasoconstriction. AT₁ receptor stimulation may also interacts with growth factor pathways, such as tyrosine phosphorylation and phospholipase C- α , resulting in activation of

downstream proteins, such as mitogen-activated protein kinase (MAPK), janus kinases (JAK), signal transducers and activators of transcription proteins (STAT), etc. These growth factor pathways, involving increased expression of early response genes, such as c-fos, c-jun, and c-myc, which are associated with cellular proliferation and growth (Sadoshima and Izumo, 1993).

Figure 1-12 Signaling pathways mediated by AT₁ receptor activation (Dinh et al., 2001).

1.7.3 Functions of AT₁ receptor activation

Major functions of AngII through AT₁ receptors are vasoconstriction, water and sodium retention, cardiac muscle contraction, growth and proliferation of cardiac and smooth muscles, and activation of sympathetic nervous system (Burnier and Brunner, 2000). AngII also has effects on lipid metabolisms in adipose tissues. It was known that, via AT₁ receptor, AngII directly inhibited lipolysis in abdominal subcutaneous adipocyte collected from normal as well as obese humans (Goossens et al., 2007). In addition, stimulation of the AT₁ receptor by AngII reduced in vitro differentiation of human pre-adipocytes, while blocking of this receptor markedly enhance adipogenesis (Janke et al., 2002a). Similarly, AT₁ receptor antagonists, telmisartan, irbesartan and losartan, induce in vitro adipogenesis of human pre-adipocytes, through increase activation of PPAR γ expression (Janke et al., 2006). AT₁ receptor activation also has important role in regulation of hormones and adipokines release from adipocytes. For example, AngII stimulates plasminogen activator inhibitor 1 (PAI-1), monocyte chemoattractant protein-1 leptin, IL-6, IL8, etc., from human and rodent primary adipocytes, while blockage of AT₁ receptor abolishes these stimulatory effects (Skurk et al., 2001; Skurk et al., 2004; Skurk et al., 2005).

1.7.4 AT₂ receptor and its signaling pathways

Like AT₁ receptor, AT₂ receptor is also a seven transmembrane domain receptor, with a molecular weight of 41 kDa, and consists of 363- amino acids. AT₂ receptor is expressed in many tissues and cells, including brain, heart, adrenal medulla, kidney, adipose tissues and adipocytes, although in lower density when comparing to the expression of AT₁ receptor (Bottari et al., 1993). However, majority of angiotensin receptors expressed in foetal organism is of AT₂ types (Grady et al., 1991). In addition, in the adult organisms, AT₂ receptor is re-expressed more in some pathological conditions, such as myocardial infarction (Nio et al., 1995), cerebral ischemia (Li et al., 2005), tissues damages (Steckelings et al., 2005), etc.

In addition to the discovery of signaling pathways involved in the AT₁ receptor, various signaling pathways are also coupled to AT₂ receptor too (Fig.1.13) (Dinh et al., 2001). For example, negative coupling of guanylate cyclase and hence reduction of cGMP level (Bottari et al., 1992), activation of various phosphatases and hence inactivation of ERK signaling (Horiuchi et al., 1997), activation of K⁺ channels while inhibition of Ca²⁺ channels (Kang et al., 1993), and activation of NO-cGMP system (Nouet and Nahmias, 2000). In addition, AT₂ receptor is also known to be linked with inhibitory G-protein, Gi protein (Zhang and Pratt, 1996).

Figure 1-13 Signaling pathways mediated by AT₂ receptor activation (Dinh et al., 2001).

1.7.5 Functions of AT₂ receptor activation

AT₂ receptor activation is known to play an important role in foetal developmental functions, since AT₂ receptor is highly expressed in foetal tissues (Dinh et al., 2001). However, even in the lower expression level of AT₂ receptor in various adult tissues, growing evidences have grown that AT₂ receptor activation also has a significant impact on body physiological homeostasis. For example, it is well known that AngII actions of neurite outgrowth and neuronal differentiation in neuronal cells are through the actions of AT₂ receptor activation (Laflamme et al., 1996; Stroth et al., 1998). In addition, AT₂ receptor activation oppositely mediates the actions of AT₁ receptor in regulation of sodium and water absorption in jejunum (Jin et al., 1998), as well as alkaline secretion in duodenum (Johansson et al., 2001). AT₂ receptor activation also mediate in blood pressure regulation, since several studies have shown that AT₂ receptor is involved in the production of cGMP, nitric oxide (NO) and prostaglandins in the kidney (Siragy and Carey, 1996, 1997a, b). Studies also suggest that AT₂ receptor mediates vasodilation which is exactly the opposite effect mediated by AT₁ receptors (Siragy, 2010). In addition, there is evidence that AT₂ receptor activation stimulates angiogenesis in vivo while AT₁ receptor mediates an inhibitory effect, suggesting that AngII mediate biological functions through two receptor subtypes with opposing actions (Walther et al., 2003).

AngII's effects on adipocytes also seem to involve through two receptor subtypes with opposing actions. AngII increases lipogenesis in 3T3-L1 and human pre-adipocytes through AT₂ receptor (Jones et al., 1997a), while AT₂ receptor deficiency suppresses adipocyte differentiation and decreases adipocyte number in atherosclerotic mice (Iwai et al., 2009). Blockage of AT₁ receptor induces human in-vitro adipogenesis, suggesting that role of AT₂ receptor on induction of adipogenesis (Janke et al., 2006). On the other hand, AT₁ receptor signaling is involved in mature adipocyte induced inhibition of pre-adipocyte differentiation (Janke et al., 2002a). The anti-adipogenic effect of AT₁ receptor in human pre-adipose cells is

also reported (Brucher et al., 2007; Fuentes et al., 2010). All these data point out the differential regulation of AT₁ and AT₂ receptor activation on adipocyte functions.

1.8 Adipogenesis

Adipogenesis is a complex process in which precursor cells differentiate into adipocytes. Although the origins of the adipose precursor cells are still poorly understood, studies on multipotent clonal cell lines have proposed that the adipocyte lineage comes from an embryonic stem cell precursor which has the capacity to differentiate into the mesodermal cell types including adipocytes, osteoblasts, and chondrocytes. During the past few years, in vitro adipocyte differentiation have been extensively studied which has led to an understanding of a clear cellular and molecular events taking place during the adipogenic process (Li et al., 2010). The most widely used in vitro cell lines for adipogenesis is 3T3-L1 and 3T3-F442A, which were clonally isolated from Swiss 3T3 cells derived from mouse embryos (Green and Meuth, 1974; Green and Kehinde, 1975).

During the growth phase, pre-adipocyte precursors are morphological similar to fibroblasts. At confluence, induction of differentiation by appropriate stimulation and condition leads to a spherical changes of cells that accumulate lipid droplets. The pre-adipocytes then progressively acquire the morphological and biochemical characteristics of mature adipocytes. Depend on the specific cell culture model or cell lines, a variety of in vitro differentiation models have been developed. Hormones and growth factors have important role in adipocyte differentiation, through their specific receptors and cascades of intracellular events which convey the external growth and differentiation signals. The combination of growth factors that trigger adipocyte differentiation is also extensively studied. Usually, agents such as insulin and glucocorticoids are necessary to induce or promote the differentiation process (Rosen and Spiegelman, 2000).

1.8.1 Transcriptional regulation of adipocyte differentiation process

To be simplified, adipogenesis process can be divided into four major stages: growth arrest, clonal expansion, early changes in gene expression and terminal differentiation (Fig.1.14) (Gregoire et al., 1998). In pre-adipocyte cell lines or primary pre-adipocytes, growth arrest appears to be required for differentiation, however, cell confluence or cell-to cell contact alone is not sufficient to undergo differentiation (Ailhaud et al., 1989). Two transcriptional factors, PPAR γ and C/EBP α , are shown to be responsible for growth arrest (Umek et al., 1991; Altioek et al., 1997). After growth arrest at confluence, if pre-adipose cells receive significant combination of mitogenic and adipogenic signals, cells continue to go through clonal expansion before differentiate into mature adipocytes. It has been shown that increase in DNA synthesis precedes expression of adipogenic markers (Amri et al., 1986). Pre-adipocyte factor (pref-1) is a recently found pre-adipocyte protein, a protein containing epidermal growth factor - like repeats. Pref-1 is involved in maintaining the phenotype of pre-adipocytes, and its expression is down regulated during adipocyte differentiation (Smas and Sul, 1993).

After growth arrest and clonal expansion, complex changes of gene expression occur. Expression of lipoprotein lipase, which plays a central role in lipid accumulation, is usually regarded as the early sign of adipogenesis (Ailhaud, 1996). Most importantly, at least two key families of transcription factors, PPARs and C/EBPs, are induced during early stage of adipocyte differentiation (Rosen et al., 1999). PPAR γ is largely adipocyte specific, although a low level of expression could be seen in pre-adipocytes. Expression of PPAR δ occurs before that of PPAR γ ; however, PPAR δ expression is not specific to adipocytes as it is detected in a variety of tissues. In addition, a transient increase in C/EBP β and C/EBP δ expression is followed by decrease in expressions in early to mid-stages of differentiation. Such decrease of C/EBP β and C/EBP δ expression is concomitant with induction of C/EBP α expression, with subsequent induction of other adipocyte specific genes. Previous studies showed that PPAR γ alone can promote adipogenesis in C/EBP α deficient cells, however, the converse has not been true (Rosen et al., 2000; Rosen et al., 2002). Furthermore, during adipocyte differentiation, cytoskeletal and extracellular matrix components also influence the expression and actions of

PPARs and C/EBPs. It has been known that decrease in actin and tubulin expression occurs during early stage of adipogenesis that precedes the expression of adipocyte-specific genes and morphological changes (Spiegelman and Farmer, 1982).

Figure 1-14 Stages of adipocyte differentiation. (Gregoire et al., 1998).

During the terminal stage of differentiation, there is increase in lipogenesis and insulin sensitivity. The protein level and activities of various enzymes involved in lipogenesis, including acetyl-CoA carboxylase (ACC), glycerol-3-phosphate dehydrogenase (GPDH), fatty acid synthase (FAS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), increase several folds (Spiegelman et al., 1983; Paulauskis and Sul, 1988). In addition, other adipocyte specific factors, such as aP2, an adipocyte specific fatty acid binding protein, glucose transporters, insulin receptor etc. also up-regulated when cells are terminally differentiated into mature adipocytes. Expressions of several adipokines, such as leptin, adiponectin, etc. are also increased during terminal differentiation of adipocytes (MacDougald et al., 1995). Evidences suggest that PPAR γ and C/EBP α are also responsible for collectively activation of

these genes, and molecular pathways underlying the induction of these adipocyte-specific genes expression have been studied extensively (Gregoire et al., 1998; Rosen and MacDougald, 2006). Taken together, these studies clearly establish the molecular and transcriptional regulation of adipogenesis. However, what kinds of external hormones and factors have effect on or how do these substances control on transcriptional factors and the adipogenic process still need to be elucidated.

1.9 Lipid droplets

Lipid droplets (LDs) are intracellular organelles that store neutral lipids within the adipocytes. LDs and circulating lipoproteins are similar in structure, in which a core of esterified lipids (triacylglycerides, cholesterol ester, retinol esters, and other ester lipids) is surrounded by a phospholipid monolayer and embedded lipid coating proteins (Ducharme and Bickel, 2008). These stored lipids can be used as basic substrates for synthesis of other important cellular structures, such as membrane phospholipids etc. In addition, triglycerides serve as a reservoir of energy that can be released as products of triacylglycerides (TAG), diacylglycerides (DAG) and free fatty acids (FFAs), in time of food scarcity. Release of FFAs from adipocytes is in one way to give energy supply for peripheral tissues. However, excess of FFA has detrimental effects on cells, often termed 'lipotoxicity'. Free fatty acids could influences several gene expressions by acting as ligands for nuclear receptors, such as PPAR family. Excess FFA is known to disrupt phospholipid membrane integrity and induce apoptosis (Mishra and Simonson, 2005). Thus, esterification of FFAs with glycerol into TAG, and then packaging of TAG into LDs provides adipocytes protected from potentially toxic metabolites, and at the same time, it can be regulated for the availability of energetic substrates for energy utilization in peripheral tissues (Greenberg et al., 2011).

Studies suggest that LDs emerge from the endoplasmic reticulum (ER) lipid bilayer (Wolins et al., 2006; Brasaemle, 2007). Ultra structural studies have shown that there is intimate association between lipid droplets and ER-like cisternal structures (Blanchette-Mackie et al.,

1995; Robenek et al., 2006; Wan et al., 2007). The close proximity of ER-like cisternae and LDs surfaces is also confirmed by transmission electron micrographs (Wolins et al., 2005). In addition, proteomic studies of lipid droplets have discovered the presence of ER related proteins, such as adipophilin etc. (Robenek et al., 2006; Wan et al., 2007). Extensive studies revealed that lipid droplets are coated with lipid droplet coating proteins of the perilipin family, including the perilipin, adipophilin (adipocyte differentiation-related protein, ADRP), tail-interacting protein of 47 kilo-Daltons (TIP47), etc., which likely control the lipid droplets metabolisms. Under resting conditions, the majority of lipid droplets are covered with perilipin, while new and smaller lipid droplets are mainly coated with S3-12 and TIP47, and larger droplets with adipophilin (Wolins et al., 2006). The heterogeneity of the lipid droplet coatings suggests the different roles of these coating proteins in lipid droplet maturation, synthesis, and breakdown of these lipid droplets.

1.9.1 Lipid droplet-coating proteins

The perilipin family of lipid droplets coating proteins is proteins covering the lipid droplets (LDs) in adipocytes that have important roles in regulation of lipid storage and utilization (Brasaemle, 2007). These proteins mainly includes perilipin, ADRP, and TIP47, and collectively known as PAT group. In addition, other new members, such as S3-12, myocardial lipid droplet protein (MLDP), oxidative tissues-enriched PAT protein (OXPAT), lipid storage droplet protein 5 (LSDP5), etc., are also regarded as the lipid droplets associated proteins.

Perilipin has two isoforms, perilipin A and B, and is the earliest known form of lipid droplet associated protein. Perilipin A and B are arising from differential splicing, while perilipin A is the major isoform found in mature adipocytes. As shown in Figure 1.15, central portion of perilipin A sequence is the hydrophobic (H1, H2, and H3) region which play a key role in targeting and anchoring perilipin to the lipid droplet's phospholipid membrane (Subramanian et al., 2004). Amino and carboxyl terminals of perilipin A are critical for facilitating TAG storage in adipocytes (Garcia et al., 2004).

Figure 1-15 Schematic diagram of perilipin A. 517- amino acid sequence of perilipin A, which includes the amino terminal of adipophilin-like domain, amphipathic β -strand and hydrophobic sequence. (Garcia et al., 2004)

Overexpression of perilipin is associated with increased TAG storage, and this increased in TAG storage is most likely due to reducing the rate of TAG hydrolysis rather than enhancing the TAG synthesis (Brasaemle et al., 2000b). Perilipin A is now regarded as essential for the hydrolysis of TAG stored in adipocytes, and is highly phosphorylated by PKA upon stimulated by lipolytic hormones. Perilipin A sequence has six consensus sites for the phosphorylation of serine residues by PKA. Phosphorylation of serine 492 triggers a major remodeling of lipid droplets causing the lipid droplets fragmentation, which allow for lipase bindings on the greater surface area of lipid droplets and hence facilitate the lipolysis (Brasaemle et al., 2009). Studies have suggested that perilipin function is mainly for stabilizing the lipid droplets. Genetic ablation of perilipin attenuates the hormone sensitive lipolysis, while the basal lipolysis rate is dramatically increased. Increased basal lipolysis was found in perilipin knockout mice which were lean and protected from genetic and diet-induced obesity (Martinez-Botas et al., 2000; Tansey et al., 2001). Proteolysis of perilipin by lysosomal protein degradation results in increasing adipocyte lipolysis (48). Low perilipin

content is also known to associate with higher lipolytic rate in human adipocytes (Mottagui-Tabar et al., 2003). Taken together, it is now well recognized that perilipin expression and its phosphorylation state play major role in regulation of adipocyte lipolysis.

Adipophilin, another major lipid droplets coating protein, is known to increase expression in the adipose tissue of perilipin knockout mice. Increased adipophilin in this adipose tissue is to replace perilipin on the lipid droplet surfaces when perilipin is absent, however, evidence suggests that it could not entirely replace the perilipin function of protection from basal lipolysis (Tansey et al., 2001). It has been known that adipophilin overexpression promotes accumulation of lipid and lipid droplets in cells, while knocking down of adipophilin reduces lipid accumulation and lipid droplets size (Imamura et al., 2002; Larigauderie et al., 2006), suggesting that adipophilin also protects the lipid droplets. However, there is no difference in lipid droplet formation and lipolysis in between adipophilin knockout and wild type mice (Sztalryd et al., 2006). That may due to observed up-regulation of TIP47 in the knockout cells, since siRNA knockout of TIP47 in these adipophilin knockout mice results in reduced lipid droplets. Taken together, these lipid droplets coating proteins could be regarded as the essential part for lipid droplets on which they directly regulate the lipolysis.

1.10 Lipolysis

Lipolysis is the catabolic process leading to the breakdown of TAGs (triacylglycerol) into NEFA (non-esterified fatty acid) and glycerol. Many lipases, mainly hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) are involved in lipolytic process (Lafontan and Langin, 2009).

Figure 1-16 Signaling pathways involved in the stimulation of lipolysis in fat cell. adipose triglyceride lipase (ATGL); adipocyte fatty acid binding protein 4 (FABP4); guanylyl cyclase (GC); monoacylglycerol lipase (MGL); type A natriuretic peptide receptor (NPR-A). (Lafontan and Langin, 2009)

1.10.1 Hormone-sensitive lipase

Hormone-sensitive lipase (HSL) is well known as the rate-limiting and key enzyme for lipolysis. Several isoforms of HSL, from 88 to 130 kDa, are produced from a single gene by different promoter activity in different tissues (98-102). HSL is made up of an N-terminal domain which interacts with FABP4 (fatty acid binding protein), and a C-terminal catalytic domain which contains the active sites as well as the regulatory sites. In vitro, HSL catalyzes the hydrolysis of TAG into DAG, and DAG into MAG (mono acyl glyceride), however, monoacylglycerol lipase (MGL) is required to obtain complete hydrolysis of MAG in vivo (Fredrikson et al., 1986). Administration of a specific inhibitor for HSL reduces plasma FFA level in animals, suggesting the in vivo role of HSL (Claus et al., 2005). In mice lacking HSL, catecholamine induced lipolysis is significantly reduced; however, basal lipolysis rate is not

much changed in these adipocytes (Haemmerle et al., 2006). The enzyme HSL also hydrolyzes cholesterol and retinyl esters (Holm, 2003). HSL is activated by cAMP and PKA, and hence catecholamines and insulin modulate HSL activity through cAMP level (Fig1.16). Three phosphorylation sites have been recognized on Ser563, Ser659, and Ser660 position of HSL. Ser659 and Ser660 are responsible for HSL activation in vitro (Anthonsen et al., 1998). It has been known that activation of ERK pathway is also responsible for activation of HSL by phosphorylating on Ser660 site (Greenberg et al., 2001). Studies also suggested that AMPK pathway is important on HSL phosphorylation, in which AICAR (a specific AMPK agonist; aminoimidazole carboxamide ribonucleotide) phosphorylates the Ser565 residue of HSL and reduces the β -agonist stimulated lipolysis in adipocytes (Sullivan et al., 1994; Daval et al., 2005).

A final regulatory step of lipolysis involves the association between HSL and FABP4 (free acid binding protein-4) which is responsible for trafficking of FFA to the plasma membrane (Fig.1.16). FABP4, also known as aP2, is a cytosolic lipid binding protein highly expressed in adipocytes. Studies suggest that FABP4 acts as a molecular chaperone facilitating FFA uptake, and also shuttles FFA out of fat cells. (59,60). It physically bind to HSL in vitro and in vivo FFA binding to FABP4 and HSL phosphorylation precedes the association of FABP4 and HSL. The association of FFA-FABP4-HSL could limit HSL activity, while docking of FABP4 to HSL favors the outflow of FFA release. Since trafficking of FFA from the site of hydrolysis at lipid droplets to the plasma membrane is necessary to complete the lipolysis, loss of FABP4 might reduce the lipolysis and FFA release. In fact, in the absence of FABP4, lipolysis is decreased, and FFA content in these adipocytes is much greater than wild-type adipocytes (Coe et al., 1999; Smith et al., 2007).

In vivo, HSL translocation from a cytosol to the surface of the lipid droplets seems to be an important step in controlling the lipolysis (Fig1.17). In basal condition, HSL is diffusively dispersed throughout the cytosolic compartment, and when stimulated with lipolytic agents, HSL moves to the lipid droplets on which it interacts with the perilipin and lipids (Egan et al., 1992; Brasaemle et al., 2000a). Absent of perilipin in adipocytes disturbed the HSL

translocation to the lipid droplets even there is increase in cAMP level (Sztalryd et al., 2003). Lipid fragmentation upon stimulated by lipolytic agents greatly increased the surface area available for HSL action of lipolysis.

Figure 1-17 Activation of HSL and perilipin through cAMP and cGMP dependent pathways. Adipose triglyceride lipase (ATGL); α/β -hydrolase domain-containing protein 5 (ABHD5); monoacylglycerol lipase (MGL); nonesterified fatty acid (NEFA); natriuretic peptide receptor-A (NPR-A); phosphorylation site in phosphorylated perilipin and phosphorylated HSL (P); triacylglycerol (TAG); diacylglycerol (DAG); monoacylglycerol (MAG) (Lafontan and Langin, 2009)

1.10.2 Adipose triglyceride lipase

Adipose triglyceride lipase (ATGL) is another important lipase capable of hydrolyzing the TAG (Fig.1.14 and 1.15). In HSL-deficient mice, ATGL is responsible for the cytosolic acyl-hydrolase activity in adipose tissue. ATGL is shown to exhibit 10 times higher specificity for TAG than DAG (Villena et al., 2004; Zimmermann et al., 2004). ATGL belongs to a family of proteins containing a patatin-like domain, and is a 486- amino acid protein with a molecular

weight of 54 kDa. A hydrophobic region from amino acid 315-360 is responsible for binding to the lipid droplets, while two phosphorylation sites were located at the C-terminal region of the enzyme (Ser404 and Ser428) (Zechner et al., 2009). It is highly expressed in adipose tissue and its expression is markedly increased during adipocyte differentiation (Mairal et al., 2006). Basal and induced lipolysis is stimulated in adipocytes overexpressed with ATAGL, while knockdown of ATGL results in decrease glycerol and FFA release (Kershaw et al., 2006). ATGL null mice show reduced adipocyte lipolysis and FFA release, and as a consequence ATGL deficient mice become obese (Haemmerle et al., 2006).

ABHD5 (α/β hydrolase domain-containing protein 5), a 349- amino acid protein with a molecular weight of 40kDa, has been shown to activate ATGL. ABHD5 is highly expressed in adipose tissues, and tightly bind to lipid droplets by interacting with perilipin-A (71, 72).

ABHD5 is able to stimulate TAG hydrolase activity in wild-type and HSL-deficient adipose tissue, but not in ATGL-deficient adipose tissue. Involvement of HSL, ATGL, and ABHD5 during lipolysis has been delineated (Lafontan and Langin, 2009; Zechner et al., 2009). During resting state (i.e., un-stimulated), HSL is distributed throughout the cytosol, whereas perilipin coating on lipid droplets is associated with ABHD5. In the stimulated stage, perilipin phosphorylation induces major remodeling of lipid droplets, and that lipid fragmentation increases the surface of lipid droplets which facilitates the action of phosphorylated HSL on TAG hydrolysis. Perilipin phosphorylation also induces the releases of ABHD5, which then binds to ATGL and hence activate the ATGL action of hydrolyzing the TAG into DAG. HSL and MGL finalized the hydrolysis of DAG into MAG. Taken together, different lipases, proteins and enzymes are involved in regulation of lipolytic process.

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell culture

2.1.1 3T3-L1 cell culture

Mouse 3T3-L1 cell line, which could differentiate into mature adipocytes upon hormonal stimulation, is one of the well-characterized and reliable in-vitro models for studying the cellular and molecular events in adipogenesis as well as in mature adipocytes (Gregoire et al., 1998; Ntambi and Young-Cheul, 2000; Fruhbeck et al., 2001). Under appropriate stimulation and incubation conditions, 3T3-L1 cells which originally derived from Swiss mouse embryo tissue by Dr. Howard Green of Harvard Medical School differentiated into an adipocyte phenotype exhibiting the morphological and biochemical characteristics, as well as proteomic expression of mature white adipocytes (Todaro and Green, 1963; Green and Meuth, 1974; Green and Kehinde, 1975; Atiar Rahman et al., 2008). When injected into the mice subcutaneous tissue, 3T3-L1 cells differentiate and form fat pads that are indistinguishable from normal adipose tissue. In vitro culture, differentiated 3T3-L1 adipocytes possess lipid droplets that mimic white adipocytes from animal tissue, as well as produce and secrete a variety of hormonal factors and adipokines (Gregoire et al., 1998).

3T3-L1 cell line used in experiments was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in growth medium (3T3-L1 pre-adipocyte medium) consisting of Dulbecco's modified Eagle's medium (DMEM), 10% (v/v) heat-inactivated calf bovine serum (CBS) and 1% penicillin-streptomycin, at 37°C in a humidified atmosphere containing 95% air and 5% CO₂ (in Forma Steri-Cycle Co₂ incubator; Thermo Scientific, Rockford, IL, USA). Cell medium was renewed every 2 days, and cells were subcultured whenever 80% confluence of cells is reached.

3T3-L1 cells (pre-adipocytes) were induced to differentiate into adipocytes as follows (Shao and Lazar, 1997; Liu et al., 2011b). After 100% confluence (regarded as day 0), cells were cultured for 2 days in DMEM supplemented with 10% fetal bovine serum (FBS), 10 µg/ml insulin, 1 µM dexamethasone (a synthetic glucocorticoid) and 0.5 mM isobutyl-1-methyl xanthine (IBMX) (3T3-L1 differentiation medium-1) (day 0 to day 2). After 48 hr incubation, the medium was replaced with DMEM with 10% FBS and 10 µg/ml insulin (3T3-L1 differentiation medium-2) (day 2 to day 4). On day 4, the cells were switched to DMEM containing 10% FBS alone (3T3-L1 adipocyte medium) (from day 4 onward), and incubation was continued for another 4 - 6 days with 2 - 3 changes of 3T3-L1 adipocyte medium. Approximately 6-7 days after induction of differentiation, 80% of cells displayed the characteristic lipid droplets-filled adipocyte phenotype. Adipocytes differentiation was assured by Oil-red-O staining and visual appearance of lipid droplets (LD).

For some experiments in Chapter 6, post-confluent 3T3-L1 pre-adipocytes were 30-min pre-treatment of 1 µM dorsomorphin and 60-min pre-treatment of 50 µM PD98059, prior to induction of differentiation. In some experiments, dorsomorphin or PD98059 or 1 µM pyroglutamated apelin-13 (Pyr-Apelin13) was continuously present during the differentiation period. These chemicals were added when the medium was refreshed every 2 days. In some experiments, 3T3-L1 pre-adipocytes were transfected with different expression vectors or siRNAs as described below, 2 – 3 days prior to induction of differentiation.

3T3-L1 pre-adipocyte medium:	DMEM high glucose, 10% CBS
3T3-L1 differentiation medium-1:	DMEM high glucose, 10% FBS, 10 µg/ml Insulin, 1µM Dexamethasone, 0.5 mM IBMX
3T3-L1 differentiation medium-2:	DMEM high glucose, 10% FBS, 10 µg/ml Insulin
3T3-L1 adipocyte medium:	DMEM high glucose, 10% FBS

2.1.2 PC12 cell culture

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla chromaffin cell, a modified post-synaptic sympathetic neuroendocrine cell which secrete catecholamines. PC12 cell line is widely regarded as in-vitro cell model for adrenal chromaffin cells. PC12 cells stop dividing and terminally differentiate into neuron cell-like phenotype (with the extension and branching of axon) if treated with nerve growth factor (NGF) for 3-4 days. Treated with dexamethasone, on the other hand, PC12 cells differentiate into more chromaffin cell-like phenotype while suppressing the expression of NGF receptors (Gunning et al., 1981; Foreman et al., 1992).

PC12 cell line used in the experiments (Chapter 4) was purchased from ATCC. Cells were cultured in growth medium (PC12 medium) consisting of advanced RPMI-1640 medium (RPMI: Roswell Park Memorial Institute), heat-inactivated 10% (v/v) FBS, 5% (v/v) horse serum, 1% penicillin-streptomycin, and maintained in 37°C incubator with 95% air and 5% CO₂. Cell medium was renewed every 2 days, and cells were sub-cultured weekly.

PC12 medium: advanced RPMI-1640, 10% FBS, 5% horse serum.

2.1.3 Co-culture system

Indirect co-culturing system allows the culturing of two types of cells simultaneously inside the two compartments separated by a microporous membrane through which the exchange of soluble factors between the two cell types occurs. Here, 'Transwell' indirect-contact co-culture system (Corning Inc., Corning, NY, USA) was used in our co-culturing experiments (Fig.2.1). In Chapter 4, 3T3-L1 pre-adipocytes and PC12 cells were cultured separately on either 6 well-plates or on transwell inserts with porous polyester membrane (0.4 µm). PC12 cells were grown, and 3T3-L1 cells were differentiated as described above. After 9-10 days of adipocyte differentiation, the inserts and wells containing different cell types were assembled, and continue to culture in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin

until further experiments. In the parallel control experiments, 3T3-L1 adipocytes or PC12 cells were separately cultured with the same conditions.

Figure 2-1 Co-culturing system with Transwell permeable-support. The picture is taken from Corning Life Science website. (http://catalog2.corning.com/lifesciences/media/pdf/transwell_guide.pdf)

2.1.4 Isolation and culturing of adipose-derived stem cells (ADSCs)

Adipose-derived stem cells (ADSC) were isolated from subcutaneous adipose tissue of the sprague-dawley rats as described previously (Zuk et al., 2001; Xu et al., 2008). Briefly, adipose tissue was washed twice with phosphate-buffered saline (PBS), cut into small pieces, and digested with 0.25% collagenase. After adding basal medium (DMEM containing 10% FBS), the suspension was centrifuged at 1000 rpm for 10 min to obtain the stromal vascular fraction. After re-suspending the pellet, cellular debris was removed through 100 μ m nylon mesh. After 24 hr incubation of the cells in the basal medium at 37°C, the non-adherent cells were removed by changing the medium, and the rat ADSCs were continuing maintained in basal medium till confluence. Subsequently, the stem cells were induced to differentiate into adipocytes for 10-14 days either in the presence or absence of 1 μ M pyr-apelin13. As previously described (Zuk et al., 2001; Kakudo et al., 2007), to induce adipogenic differentiation, the ADSCs were cultured in the adipogenic medium, that is, DMEM

containing 10% FBS supplemented with a hormonal cocktail (10 $\mu\text{g/ml}$ insulin, 1 μM dexamethasone, 0.5 mM IBMX, and 200 μM indomethacin). The medium was refreshed every 2 - 3 days.

For long-term preservation, 3T3-L1 cells or PC12 cells were detached and re-suspended in freezing medium containing 95% of respective growth medium supplemented with 5% dimethyl sulfoxide (DMSO), followed by transferring the cells suspension into freezing vials which then stored in liquid N_2 . To recover the cells, preservation was immediately transferred from liquid N_2 to a 37°C water-bath, and then adds two volumes of respective growth medium after it was thawed. Cells suspension was then centrifuge at 1200 rpm (MIKRO 22R, Hettich Zentrifugen, Tuttlingen, Germany) for 5 min, followed by removal of supernatant and addition of respective growth media to re-suspend cell pellet, and then finally maintained in culture conditions as described.

All media and supplements are from Gibco BRL (Invitrogen, Carlsbad, CA, USA). Experiments were always conducted in parallel using the same batch of cells. All media contained 100 U/ml of penicillin and 10 $\mu\text{g/ml}$ of streptomycin.

2.2 Chemicals and solutions

Bath-solution contains (in mM): 150 NaCl, 5.4 KCl, 2 MgCl_2 , 2 CaCl_2 , 5 mM glucose, 10 HEPES (4-(2-hydroxyethyl-1-piperazine ethane sulfonic acid); titrated to pH 7.4.

High K^+ solution (in mM): 40 NaCl, 105 KCl, 1 MgCl_2 , 6 CaCl_2 , 10 HEPES; pH 7.4

Zero Ca^{2+} solution (in mM): 150 NaCl, 5.4 KCl, 2 MgCl_2 , 5 Glucose, 10 HEPES; pH 7.4

Dopamine loading buffer (in mM): 68 NaCl, 2.5 KCl, 1 MgCl_2 , 2 CaCl_2 , 10 HEPES, 1.5 Ascorbate, 70 Dopamine; pH 7.2. Ascorbate and dopamine was added shortly before cell loading.

Tris-buffered saline buffer (TBS) (in mM): 10 Tris, 150 NaCl; pH7.4.

Tris-buffered saline-Tween (TBST) buffer (in mM): 10 Tris, 150 NaCl and 0.05% Tween-20; pH7.4.

RIPA lysis buffer: 1x TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide

Complete RIPA lysis buffer: 1x RIPA lysis buffer, 10 μ l PMSF (phenylmethanesulfonylfluoride; a serine protease inhibitor), 10 μ l sodium orthovanadate (inhibitor of protein tyrosine phosphatases, alkaline phosphatases), 10-20 μ l protease inhibitors cocktail solution.

Chemicals

8-bromo-cGMP (8-Bromoguanosine cyclic 3',5'-monophosphate sodium salt):

Membrane permeable cGMP analog that activates protein kinase G (PKG)

BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester): Cell-permeable and selective calcium chelator

Brefeldin-A (1,6,7,8,9,11a β ,12,13,14,14 α -Decahydro-1 β ,13 α -dihydroxy-6 β -methyl-4H-cyclopent(f) oxacyclotridecin-4-one): Reversible inhibitor of protein translocation from the endoplasmic reticulum (ER) to the Golgi apparatus.

Calcimycin (5-(Methylamino)-2-[[2R,3R,6S,8S,9R,11R]-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-(1H-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazolecarboxylic acid):

Calcium ionophore that increase intracellular calcium concentration

CN/DAB (4-chloro-1-naphthol / 3,3'-diaminobenzidine tetrahydrochloride)

Cyclohexamide (4-[2-(3,5-Dimethyl-2-oxo-cyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione): Selective inhibitor of eukaryotic protein synthesis

DMSO (Dimethyl sulfoxide)

Dorsomorphin dihydrochloride (Compound C; 6-[4-[2-(1-Piperidinyl)ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine dihydrochloride): Potent and selective inhibitor of AMP-activated protein kinase (AMPK)

EDTA, Ethylenediaminetetraacetic acid (N,N'-1,2-Ethanediybis[N-(carboxymethyl)]glycine):
Chelating agent; sequesters di- and trivalent metal ions; Calcium chelator

EGTA (Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid): Calcium chelator

Forskolin or ([3R-(3 α ,4 α ,5 β ,6 β ,6 α ,10 α ,10 α ,10 β)]-5-(Acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1H-naphtho[2,1-b]pyran-1-one):
Cell-permeable activator of adenylyl cyclase

Fura-2-AM (Fura-2-acetoxymethyl ester):
A membrane-permeable derivative of the ratiometric calcium indicator Fura-2

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid):
A zwitterionic organic chemical buffering agent

H89 dihydrochloride (N-[2-[[3-(4-Bromophenyl)-2-propenyl] amino] ethyl]-5-isoquinoline sulfonamide dihydrochloride): Protein kinase A inhibitor

KT5720((9R,10S,12S)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, hexyl ester): Potent, selective inhibitor of protein kinase A

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide):
A pale yellow chemical that is cleaved by living cells to yield a dark blue formazan product

PD98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one):
Selective inhibitor of mitogen-activated protein kinase kinase (MAPK kinase)

PD 123319 ditrifluoroacetate (1-[[4-(Dimethylamino)-3-methylphenyl] methyl]-5-(diphenyl acetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate):
A potent, selective, non-peptide angiotensin AT2 receptor antagonist

Ro 32-0432 hydrochloride (3-[(8S)-8-[(Dimethylamino) methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-10-yl]-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione hydrochloride):
Selective cell-permeable protein kinase C inhibitor

ZD 7155 hydrochloride (5,7-Diethyl-3,4-dihydro-1-[[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]-1,6-naphthyridin-2(1H)-one hydrochloride):

A potent and selective competitive antagonist for the angiotensin II type 1

Dorsomorphin, KT5720, PD98059, PD123319, ZD7155, Ro32-0432, 8-bromo-cGMP were purchased from Tocris Bioscience (Ellisville, MO, USA). Pyr-Apelin13 (cat no# sc359030) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Leptin (mouse leptin; cat no#L3772) was purchased from Sigma (Sigma-Aldrich, Saint Louis, MO, USA), and resistin (murine resistin; cat no#4560-25) from Biovision (Mountain View, CA, USA). Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich.

2.3 Plasmids and siRNAs

The cDNA of murine resistin in the pCMV6 vector (cat no# MC204783, Mus musculus resistin (Retn)), and cDNA of Apelin-tGFP in pCMV6 vector (cat no# MG200116, GFP-tagged of Mus musculus apelin (Apln)) were purchased from OriGene Technologies (Rockville, MD, USA). The vector pEGFP-N1 (cat no# 6085-1) was purchased from Addgene (Cambridge, MA, USA).

The plasmid encoding murine leptin cDNA was a kind gift from Prof. Jan Tavernier (Ghent University, Belgium), the Glut4-GFP plasmid (glucose transporter – 4) from Prof. Jeffrey E Pessin (Albert Einstein College of Medicine, USA), the Rab5-GFP from Dr Sean Liour (Institute of Bioengineering and Nanotechnology, Singapore), and the NPY-EGFP (NPY: Neuropeptide Y, EGFP: enhanced GFP) from Prof. Wolf Almers (Vollum Institute, Oregon Health Science University).

The Resistin-EGFP/Leptin-EGFP plasmids were constructed by sub-cloning the corresponding cDNAs into the pEGFP-N1 vector at the N-terminal of EGFP between the Nhe I and AgeI sites, and these insertions were verified by DNA sequencing.

Mouse Apelin-siRNA (cat no#sc44742), APLNR-siRNA (cat no#sc44733) and control siRNA were purchased from Santa Cruz Biotechnology.

2.4 Transient transfection

3T3-L1 pre-adipocytes at concentration of 2×10^5 were sub-cultured on sterilized 25 mm (diameter) high refractive-indexed cover-glass (No1, Paul Marienfeld GmbH, Germany) pre-coated with 0.01% PLL (poly-L lysine, Sigma), and 2 days after confluent, cell were differentiated as described above. Six to seven days after initiation of differentiation, 3T3-L1 adipocytes were transfected with 2 - 5 μ g of relevant plasmids, such as leptin-EGFP, resistin-EGFP, apelin-tGFP, Rab5-GFP, or Glut4-GFP, using FuGene6 Transfection reagent (Roche Diagnostics GmbH, Indianapolis, IN, USA), followed by another 1 – 3 days of culturing in normal growth medium (3T3-L1 adipocyte medium) before the imaging experiments.

PC12 cells at concentration of 2×10^5 were sub-cultured on PLL coated cover-glass, and then transfected with NPY-GFP, using FuGene6, followed by 1 - 3 days culturing in PC12 growth medium before imaging experiments to achieve the highest expression of GFP tagged fusion protein.

For experiments in Chapter 6, following transfection protocol was used (Hashimoto et al., 2009). Briefly, one day after being split, 3T3-L1 cells were incubated with OPTI-MEM (Gibco) containing Lipofectamine2000 transfection reagent (0.5% v/v) (Invitrogen) with relevant siRNAs (20 nM) or plasmids (5 μ g) for 4 hr, followed by adding with DMEM containing 20% CBS, and incubation proceeded for additional 8 - 12 hr. The medium was then replaced with normal growth medium (3T3-L1 pre-adipocyte medium), and when two days after confluence, differentiation was induced as mentioned above.

Overexpression or knocking down of gene expression was confirmed by immunoblot analyses of protein production or secretion in transfectant cells.

2.5 Oil red O staining

Oil red O is a lysochrome (fat soluble dye) widely used for staining of neutral triglycerides inside the lipid droplets. Hence, staining lipid droplets inside adipocytes and/or quantification of Oil red O staining can be considered used as the indicator for the degree of adipocyte differentiation.

After 6 - 9 days of adipocyte differentiation, the formation of lipid droplets in cells was analyzed as follows (Hashimoto et al., 2009; Liu et al., 2011b). After removing of cell medium, cells were washed 2 times with phosphate buffered saline (PBS) and fixed with 10% formalin in PBS at room temperature for 10 min. Then, the cells were incubated with a filtered Oil red O staining solution (1.8 mg/ml of Oil Red O and 60% v/v isopropanol) for 15 - 30 min at room temperature followed by washing twice with distilled water. Cells were kept in water and imaged under an optical microscope. Fat droplets in adipocytes were stained as red color (Fig.2.2). In some experiments (Chapter 6), Oil red O was extracted using 100% isopropanol and absorbance was determined using a spectrophotometer at a wavelength of 520 nm.

Figure 2-2 Typical Oil red O staining of lipid droplets inside the differentiated 3T3-L1 adipocytes.

2.6 Confocal microscopy

Confocal microscopy offers many advantages over conventional fluorescence microscopy, including the elimination of out-of-focus glare, and hence gives excellent optical resolution and contrast of the image. It can collect the light reflected or emitted by a single plane of the specimen when a laser beam is scanning the specimen pixel by pixel and line by line (Fig.2.3). In addition, a number of images generated with the focal plane shifted can be combined into a 3-dimensional image stack. Laser scanning confocal (LSM) microscopy is currently the most widely used confocal microscopy variations in biomedical research field. Here, LSM 510 Meta (Carl Zeiss) was used to analyze the distribution and localization of various molecules, adipokines and receptors in adipocytes.

3T3-L1 adipocytes (day 6 - 9) grown on the PLL coated glass-coverslips were fixed with 4% formaldehyde for 10 – 20 min, and then permeabilized with 0.1% TritonX-100 for 10 min. Non-specific binding sites were then blocked with 1.5% bovine serum albumin (BSA) for 1 hr, prior to overnight incubation of specific primary antibodies (1: 50 - 100 dilution), followed by PBS wash and subsequent incubation with respective secondary antibodies (1: 200 - 400 dilution) for 2 – 3 hrs. Specifically,

Figure 2-3 Principle of confocal microscopy. The picture is taken from Olympus microscopy website. (<http://www.olympusfluoview.com/theory/index.html>)

-Leptin molecules were immunostained first with chicken anti-leptin IgY antibody (Abcam, Cambridge, MA, USA; cat no#ab17531) or rabbit anti-leptin IgG antibody (Sigma, cat no#L3410), and subsequently with the anti-chicken IgY secondary antibody conjugated with FITC (Fluorescein isothiocyanate) (Abcam; cat no#ab46969) or anti-rabbit IgG secondary antibody conjugated with Atto 647 NHS ester (Sigma, cat no#40839).

-Resistin molecules were immunostained first with rabbit anti-resistin IgG antibody (Biovision; cat no#5999-100) and subsequently with the anti-rabbit IgG secondary antibody conjugated with Atto 647 NHS (Sigma).

-Perilipin was immunostained first with goat anti-perilipin IgG antibody (Santa Cruz; cat no#sc33469) and subsequently with the anti-goat IgG secondary antibody conjugated with FITC (Sigma; cat no#F7367).

-Apelin molecules were immunostained first with goat or rabbit anti-apelin IgG antibody (Santa Cruz; cat no#sc33469) and subsequently with the anti-goat IgG secondary antibody conjugated with FITC or anti-rabbit IgG secondary antibody conjugated with Atto 647 NHS (Sigma).

-APJ receptors (apelin receptor) were immunostained first with a rabbit anti-apelin receptor IgG (Santa Cruz; cat no#sc33823), and subsequently with anti-rabbit IgG secondary antibody conjugated with Atto647 NHS (Sigma).

In co-localization study, leptin and resistin, or apelin and APJ receptor, were co-immunostained with respective primary and secondary antibodies. In some experiments (Chapter 3 and 6), the adipocytes were transfected with plasmids encoding Glut4-GFP, Rab5-GFP, or apelin-tGFP, 3 – 4 days before fixation. In some experiments (Chapter 6), adipocytes were pre-incubated without or with Pyr-apelin13 and/or epinephrine for 4 hr at 37° C before fixation.

After staining and washing with PBS, adipocytes were analyzed using a Confocal Laser Scanning Microscope (LSM 510 Meta), equipped with 63x oil-immersion objective (1.4 numerical aperture) (Carl Zeiss GmbH, Jena, Germany), and images were acquired and processed with Zeiss LSM image browser software.

2.7 Total internal reflection fluorescence microscopy (TIRFM)

Total internal reflection fluorescence microscopy (TIRFM) imaging and analyses is the key instrumental to reveal the trafficking and fusion of secretory vesicles (fluorescently-labeled) in the subplasmalemmal region. Unlike the conventional fluorescence microscopy, TIRFM provide the unique evanescent waves that selectively illuminate and excite the fluorophores in the thin membrane region (<200 nm) immediately adjacent to a glass-water interface (glass-bath solution interface) (Fig.2.4). TIRFM is based on the concept that, at a specific critical angle, the laser beam is totally reflected from the glass-water interface in accordance with Snell's Law (Fig.2.4). That reflection generates a very thin electromagnetic field (evanescent wave or field) which undergoes exponential intensity decay with increasing distance from the glass-water interface. Therefore, using TIRFM, events like fusion of (fluorescently-tagged) vesicles with the plasma membrane, or trafficking of (lateral or vertical motion) (fluorescently-tagged) vesicles just under the plasma membrane, could be detected with high temporal resolution and contrast.

Figure 1

Figure 2-4 Principle of TIRF microscopy. The picture is taken from Olympus microscopy website. (<http://www.olympusmicro.com/primer/techniques/fluorescence/tirf/tirfintro.html>)

TIRFM experiments were achieved using a Zeiss Axiovert-200M inverted microscope system (Carl Zeiss, Göttingen, Germany) which is equipped with a 100x 1.45 NA TIRF objective lens. The glass-coverslip (refractive index=1.52), on which 3T3-L1 adipocytes transfected with leptin-EGFP, or resistin-EGFP, or apelin-tGFP, or PC12 cells transfected with NPY-GFP were grown, was mounted in an imaging chamber (Bioscience tools, San Diego, CA, USA) filled with the bath-solution. The laser beam (488 nm) was pointed to the coverslip at 68-70° from normal. The penetration depth of the evanescent field was about 100 nm. The emission of EGFP or tGFP was collected at 520 nm. Using MetaMorph 6.3 (Molecular Devices, Downingtown, PA, USA), time-lapse digital images were attained from a single cell by an EMCCD (electron multiplying charge coupled device) camera with exposure times of 25 ms and 2 - 3 Hz sampling for 2 min. Pixel size of the digital images is 0.248 μm . Motions of individual vesicles were tracked using Image J (National Institute of Health, Bethesda, MD, USA), and analyzed by Igor (WaveMetrics, Lake Oswego, OR, USA) routines.

Large dense core vesicles (LDCV) were specifically labeled by overexpressing NPY-EGFP in PC12 cells; and leptin-, resistin-, and apelin- vesicles in 3T3-L1 adipocytes were labeled by overexpressing leptin-EGFP, resistin-EGFP, and apelin-tGFP respectively (GFP is tagged at the C-terminals to make sure that the signal peptide at the N-terminals are free for endogenous localization) .

Lateral movement of the secretory vesicles can be considered as random Brownian motion confined within a limited range by certain physical barrier or tethering forces. Vesicles also travel vertically (vertical movement), i.e., they arrive from the cytoplasmic compartment to the subplasmalemmal compartment and retrieve back after some dwelling time (the time during which vesicles dwelling under subplasmalemmal region). Average velocity of the vesicle was defined as the total distance of vesicle lateral movement (parallel to the plasma membrane) divided by vesicle dwell time (Zhang et al., 2008). Rate of vesicles arrived from cytosol to the subplasmalemmal region was regarded as the vesicle vertical motion. Fusion event of the vesicle (Fig.2.5 and 2.6) is characterised and regarded if there is 1) sudden rise of fluorescence intensity about the vesicle centre owing to rapid release of fluorescent molecules, 2)

accompanying increase of fluorescent intensity in the peripheral region because of the diffusion of released molecules, and 3) final fading of the vesicle (Bai et al., 2007).

Statistics were showed in mean $\pm$ standard error of the mean (SEM), and p values were calculated by Student's unpaired t -test, or Mann-Whitney rank sum test for skewed distribution of vesicle velocity.

Figure 2-5 Consecutive time-frames showing a typical fusion event of GFP-tagged resistin vesicle was generated from a stack of 10 time-lapse images at a frame rate of 3 Hz. Note the increase of fluorescence intensity followed by diffusion. (Hz: hertz, number of time-lapse images taken per second).

Figure 2-6 A kymograph depicting the docking and fusion event of individual GFP-tagged leptin vesicle. Kymograph was generated from a stack of 240 time-lapse images at a frame rate of 3 Hz. (Kymograph: a graphical representation of spatial position over time in which a spatial axis represents time)

2.8 Carbon fiber amperometric recording and analyses

Exocytosis of secretory vesicles can be detected by amperometric recording using a carbon fiber microelectrode (CFE) (Mosharov and Sulzer, 2005; Mosharov, 2008). A CFE held at a positive potential (~ 700 mV) is placed against the cell membrane (Fig.2.7). Exocytosis is then observed as amperometric spikes: electrochemical current produced by the transfer of electrons after oxidation of secreted electroactive molecules (e.g., catecholamines). Each spike represents a single exocytotic event, and measurement of spike frequency provides information about the quantity of vesicle release. In addition, spike parameters or kinetics also allows us to observe the real-time kinetics of exocytotic event. Here, amperometric recording was also used to test the effect of adipokines (leptin and resistin) on the secretion of catecholamines from LDCVs of PC12 cells.

Exocytosis of catecholamines from dopamine-loaded PC12 cells was induced by local delivery of stimulation solution (high K^+ solution) through a fine glass micropipette (tip size of 2 – 3 μm) placing ~ 5 - 10 μm away from the cell membrane by the Burleigh manipulator. The micropipettes were obtained by pulling the B100-50-10 borosilicate glass capillaries (O.D.: 1.0 mm, I.D.: 0.50 mm, length: 10 cm) (Sutter Instrument Co., Novato, CA, USA), using a P-2000 Laser Based Micropipette Puller (Sutter). The carbon-fiber-microelectrodes (CFEs, 5 μm) were purchased from ALA Scientific Instruments (Westbury, NY, USA), and the tip was cut to expose a fresh surface before every recording for consistent sensitivity. Amperometric current recording was performed by an EPC-10 double patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) using a 5- μm CFE, which was placed gently on the cell membrane. For testing the effect of leptin or resistin on catecholamine release, PC12 cells were pre-incubated for 30-min in the bath solution without or with 50 nM leptin or 50 nM resistin before recording. For each recording, carbon fiber tip was freshly cut and then gently placed near the cell membrane. While the cell was stimulated with high K^+ solution, the amperometric signals were recorded for 2-min with 4 kHz sampling rate and 1 kHz filtering frequency. Amperometric signals were examined by a Igor program, Amperometric Spike Analysis 8.15, developed by Dr. Eugene Mosharov (Department of Neurology, Columbia University). Amperometric spikes with amplitudes less than 2 pA (5 times of the background noise) were regarded as noise and omitted for analysis. Statistics were showed in mean $\pm$ SEM, and p values were calculated by Student's t -test.

Figure 2-7 Demonstration of the Amperometric Recording. Freshly-cut carbon fiber electrode (black) is gently positioned on the cell membrane while a glass pipette locally delivers the stimulation solution. The secreted electroactive molecules (e.g., catecholamines) could be oxidized and produced a current spike which reflects the kinetics of quantal vesicle release. (Soo et al., 2010)

2.9 [Ca²⁺]_i imaging and analyses

The level of intracellular calcium concentration ([Ca²⁺]_i) was reported by a membrane-permeable and Ca²⁺ sensitive dye, Fura-2-AM (Fura-2 acetoxy-methyl ester) (Molecular Probes, Eugene, OR, USA), which allows the real time recording of [Ca²⁺]_i in live cells (Tsien, 1984). A ratiometric Fura-2-AM dye allows for [Ca²⁺]_i calculation based on a ratio of measurements made with two excitation wavelengths. Fura-2 undergoes a shift in excitation spectrum upon Ca²⁺ binding (Fig.2.8).

Figure 2-8 The excitation spectrum of fura-2-AM (Molecular Probes, Eugene, OR, USA).

The $[Ca^{2+}]_i$ concentration in Fura-2-AM loaded cell can be determined by measuring the ratio of two fluorescence emissions at 510 nm in response to two excitation wavelengths (340 nm and 380 nm). Hence, this measurement is only reliant on Ca^{2+} concentration but not on the absolute fluorescence amount or other background signals such as auto-fluorescence.

3T3-L1 adipocytes or PC12 cells grown on the PLL-coated glass-coverslips were loaded with 5 μ M Fura-2-AM for 40-60 min in respective culture medium at 37°C. Then, the cells were rinsed and rested for another 30-min to let de-esterification and retention of membrane permeable Ca^{2+} dye, Fura-2-AM.

For $[Ca^{2+}]_i$ imaging of PC12 cells (for experiments in Chapter 4), cells were then incubated for 30-min in the bath solution without or with 50 nM leptin or 50 nM resistin before recording. $[Ca^{2+}]_i$ was recorded as the ratio of emissions at 510 nm resulting from the excitation of 340 nm and 380 nm (F340/F380). Fluorescence signals in response to 2-min perfusion high K^+ solution were obtained from single cell using a photometry system, which include a Polychrome V monochromator (TILL Photonics GmbH, Gräfelfing, Germany).

For $[Ca^{2+}]_i$ imaging of 3T3-L1 adipocytes (for experiments in Chapter 3), GPI (25 mM glucose, 1mM pyruvate, 100 ng/ml insulin), 10 μ M forskolin, 100 nM leptin/resistin, or 1 mg/ml calcimycin was added in the bath solution while recording the $[Ca^{2+}]_i$ signal. For $[Ca^{2+}]_i$ imaging of 3T3-L1 adipocytes (for experiments in Chapter 5), cells were incubated in zero- Ca^{2+} bath solution, without or with 1 μ M PD123319 for 30 min before the imaging experiments. Fluorescence signals were recorded as described above, and after recording a stable baseline, the adipocytes were induced with AngII (100 nM) solution. Actual $[Ca^{2+}]_i$ level were calibrated using a protocol described by Williams and Fay (Williams and Fay, 1990).

2.10 Western blot analyses

Western blots were mainly used to analyze the expression and secretion of adipokines, and expression of other proteins, enzymes, and receptors.

3T3-L1 pre-adipocytes were grown till confluence, and induced to differentiate as described above. In Chapter 3, on day 10 or 11 of differentiation, adipocytes were washed with PBS for three times, and incubated at 37°C in serum-free medium (Lonza, Basel, Switzerland) containing 5.5 mM glucose without (control) or with addition of GPI (1 mM pyruvate, 20 mM glucose, 100 ng/ml insulin), or GPI and 10 µM forskolin, or 10 µM forskolin, or 100 nM leptin, or 100 nM resistin, for 2 hr. In some experiments, the cells were pre-incubated with intracellular calcium chelator BAPTA-AM (50 mM) in the Ca²⁺-free bath solution for 15 min to deplete intracellular Ca²⁺. In some experiments, 10 mM EGTA was added in the serum-free medium to deplete extracellular Ca²⁺. In Chapter 4 and 5, after 8 – 11 days of differentiation, adipocytes were washed with PBS, and incubated in serum-free DMEM medium with or without testing chemicals for indicated period of time at 37°C.

The media were then harvested, and stored at -20°C after being concentrated using AmiconUltra centrifugal filter units (Millipore, Billerica, MA, USA) for immunoblot analyses of adipokine secretion. After washing the cells with ice-cold PBS for three times, a cell lysate was collected using RIPA lysis buffer containing a cocktail of protease inhibitors (Santa Cruz Biotech). In some experiments (Chapter 6), 3T3-L1 pre-adipocytes were lysed using RIPA lysis buffer, during differentiation process at indicated time, for analyses of protein production during adipogenesis. These whole cell samples were vortexed briefly and then centrifuged for 5 min at 14,000 rpm at 4°C.

Cells were grown confluent in the flasks of same size, and all the western blot experiments under different conditions were performed in parallel (same time and the same batch of cells). Hence, the cell number for each experiment is almost the same. In addition, proteins concentrations in the harvested culture medium or the supernatant of the cell lysate were verified using a protein assay kit (Bio-Rad Lab, San Diego, CA, USA) based on Bradford method. This was used to control the sample volume for the immunoblotting experiments to minimize the cell number variation. Furthermore, intracellular GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as an indicator of the total cell number was also quantified and used as the internal loading control.

Protein samples were separated on the 10-12% SDS-polyacrylamide gels using a Mini-Protean Tetra cell followed by transblotted onto the nitrocellulose or polyvinylidene fluoride (PVDF) membrane at 4°C with the help of a Mini-Transblot apparatus (Bio-Rad Lab). Membrane was then incubated for 2 hr in blocking solution: 5% skimmed fat milk or 5% BSA in Tris-buffered saline-Tween (TBST) buffer. Membrane was subsequently incubated for 12 hr at room temperature with specific primary anti-mouse IgG antibodies (1:200-400 dilutions) in TBST: anti-leptin (Ob; cat no#sc9014; cat no#L3772-Sigma), anti-resistin (cat no#5997-100, #5999-100; Biovision), anti-apelin (cat no#sc33469), anti-apelin receptor (APLNR; cat no#sc33823, #sc33838), anti-PPAR α (cat no#sc7196), anti-phospho-PPAR α (cat no#sc28001), anti-C/EBP α (cat no#sc61), anti-perilipin (cat no#sc47319), anti-phospho-perilipin (cat no#4856; Vcell Science), anti-aP2 (A-FABP; cat no#sc18661), anti-FAS (cat no#sc20140), anti-ACC (cat no#3662s; Cellsignaling), anti-Wnt10b (cat no#sc25524), anti-pERK (cat no#3441-100; Biovision), anti-ERK (cat no#3085-100; Biovision), anti-GAPDH (cat no#9545; Sigma), anti-GFP (cat no#3999-10; Biovision), or anti-NPY antibodies. (Unless otherwise stated, all antibodies were purchased from Santa Cruz). After washing for 3 times with TBST buffer, membrane was then incubated with respective horseradish peroxidase-conjugated anti-IgG secondary antibodies (1:2000-5000 dilutions) for 6-8 hr at room temperature. The protein bands were detected with CN/DAB detection kit (Thermo Scientific) or Opti-CN detection kit (Bio-Rad), and quantified using Bio-Rad's Densitometer. Results are reported as mean $\pm$ SEM, and the statistical significance was analyzed by Student's unpaired *t*-test.

Anti-mouse primary antibodies against apelin, apelin receptor, PPAR α , phospho-PPAR α , C/EBP α , perilipin, aP2, FAS and Wnt10b were purchased from Santa Cruz Biotech. Anti-mouse primary antibodies against leptin, resistin, NPY, GFP, ERK and pERK were purchased from Biovision. Primary antibodies against mouse -ACC and -perilipin were purchased from (Cell Signaling Technology Inc., Beverly, MA, USA). Anti-mouse phospho perilipin antibody was purchased from (ValaScience Inc., San Diego, CA, USA). Anti-mouse leptin and GAPDH

were purchased from Sigma. Horseradish peroxidase-conjugated secondary antibodies were purchased from Thermo Scientific and Sigma.

2.11 ELISA or EIA analyses

Enzyme linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) were used to analyze the secretion of catecholamines from PC12 cells or to test the secretion or expression of adipokines in adipocytes.

For detection of catecholamine secretion (in Chapter 4), firstly, PC12 cells were grown in the 25 cm² flasks (3 x 10⁶ cells / flask) as described above till confluence. After washing with PBS, cells were incubated for 1 hr in serum and phenol-red free RPMI-1640 media without or with testing chemicals at 37°C. The culture media were then collected, and added with 1 mM EDTA and 4 mM sodium metabisulfite in order to prevent the catecholamines degradation, and then immediately frozen at -80°C. Epinephrine concentration was quantified using Epinephrine ELISA kit (Immuno-Biological Lab, Minneapolis, MN, USA; cat no#IB89539).

For testing the apelin secretion from adipocytes, firstly, 3T3-L1 cells were grown and differentiated as described above. 8-9 days of adipocytes differentiation, adipocytes were washed three times with PBS, and incubated in the serum-free medium (equal amount) with or without the testing chemicals for a defined period of time at 37° C. In some experiments, adipocytes were pre-incubated with PD98059 or Ro32-0432 for 60 min and 30 min respectively, prior to incubation of the cells with AngII and/or AngII receptor antagonists. In some experiments, cells were co-incubated with 8-bromo-cGMP or forskolin, plus AngII and/or AngII receptor antagonists. Protein concentration (an indicator of the total cell number) was essentially identical for all samples, since equal amount of medium were collected from the confluent cell cultures. Medium samples with the same protein concentration were used for EIA analysis. Apelin concentration in collected cultured medium was quantified using apelin C-terminus EIA kit (RayBiotech Inc., Norcross, GA, USA; cat no#EIA-APC-1). Results are

reported as mean $\pm$ SEM, and the statistical significance was analyzed by Student's unpaired *t*-test.

2.12 Adipogenesis assay

Adipogenesis assay was used to quantify the cellular triglyceride content in adipocytes. Adipogenesis assay was determined by using an Adipogenesis Assay kit (Biovision Inc; cat no#K610-100) according to the manufacture's protocol. Briefly, 3T3-L1 adipocytes (on day 6 - 7) cultured in 6-well plates were washed twice with PBS, scraped and homogenized the samples, and then assayed for total triglyceride. Colorimetric assay was then performed at 570 nm in a plate reader (Victor3). The protein concentrations from these adipocytes were used as internal control, and measured with a protein assay kit (Bio-Rad). Results are reported as mean $\pm$ SEM, and the statistical significance was analyzed by Student's unpaired *t*-test.

2.13 MTT cell viability and proliferation assay

The viability of cells after different treatments or transfections was determined by using a MTT cell growth determination kit (Sigma). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide), is a pale yellow chemical that is cleaved by living cells to yield a dark blue formazan product, which can be measured with a spectrophotometer. In brief, on the testing day, after washing the cells with PBS, MTT reagent solution (5mg/ml MTT in RPMI-1640 without phenol red) with the amount 10% of culture volume was added to each well, and then incubated for 4 hr at 37°C. Then, solubilizing buffer, MTT solvent (0.1 N HCL in anhydrous isopropanol) with the amount equal to the culture volume was added to each well. Within one hour of incubation, the absorbance was then measured in an ELIAS-type plate reader (Victor3) at a wavelength of 570 nm, while background absorbance was measured at

690 nm to be subtracted. Results are reported as mean $\pm$ SEM, and the statistical significance was analyzed by Student's unpaired *t*-test.

2.13 Measurement of free fatty acids (FFA)

3T3-L1 adipocytes were incubated in serum-free DMEM for 4 hr at 37° C, and FFA content in the media was determined with a free fatty acid quantification kit (Biovision; cat no#K612-100) according to the manufacturer's protocol. In some experiments, adipocytes were incubated in DMEM with exposed to pyr-apelin13 and/or epinephrine for 4 hr before FFA measurement. Optical density at 570 nm was used for colorimetric assay (Victor3, PerkinElmer), and all experiments were carried out in duplicate. Results are reported as mean $\pm$ SEM, and the statistical significance was analyzed by Student's unpaired *t*-test.

CHAPTER 3 VESICULAR STORAGE, VESICLE TRAFFICKING, AND SECRETION OF LEPTIN AND RESISTIN: THE SIMILARITIES, DIFFERENCES AND INTERPLAYS

3.1 Introduction

Adipose tissue, which was previously regarded as a passive depot of excess energy (fat), has recently been recognized as the largest and a highly active endocrine organ secreting a spectrum of signaling molecules called adipokines (including leptin, resistin, adiponectin, visfatin, TNF- α , etc.). These adipokines play key roles in regulating energy homeostasis, lipid metabolism, immunity, and other physiological functions (Rosen and Spiegelman, 2006; Lago et al., 2007). Among them, leptin and resistin have attracted much interest because of their critical involvement in metabolic disorders (Rondinone, 2006; Rosen and Spiegelman, 2006).

There are evidences that the functions of leptin and resistin as well as their secretion are intimately linked. For instance, the secretion of leptin and resistin is similarly controlled by diet. Fasting reduces the serum levels of both adipokines, while food intake does the opposite (Kolaczynski et al., 1996; Rajala et al., 2004). Both adipokines inhibit differentiation of adipocytes and thus development of adipose tissue (Zhou et al., 1999; Dal Rhee et al., 2008). They all encourage angiogenesis and actively regulate immune responses (Bouloumie et al., 1998; Reilly et al., 2005; Mu et al., 2006; Lago et al., 2007; Anagnostoulis et al., 2008). On the other hand, they antagonize each other in some cases (Rondinone, 2006). For instances, leptin increases insulin sensitivity whereas resistin suppresses it (Ogawa et al., 1999; Stepan et al., 2001a); leptin reduces gluconeogenesis whereas resistin promotes it (Rossetti et al., 1997; Banerjee et al., 2004); leptin enhances AMPK mediated signal transduction while resistin decreases it (Minokoshi et al., 2002; Palanivel and Sweeney, 2005).

The mechanisms of leptin and resistin secretion, and the cross-talks between them, however, are poorly elucidated. Based on confocal fluorescence microscopy, total internal reflection fluorescence microscopy (TIRFM), and immunoblotting, we aim to study the compartmentalization and the regulations of vesicular trafficking and secretion of both leptin and resistin, using adipocytes differentiated from 3T3-L1 fibroblasts. The commons, differences, and interplays between these two important adipokines are revealed.

3.2 Compartmentalization of leptin and resistin

We studied the compartmentalization of both leptin and resistin molecules using 3T3-L1 derived adipocytes. Based on immunostaining with adipokine-specific antibodies, confocal fluorescence microscopy revealed that both leptin and resistin molecules were segregated into numerous small vesicles throughout the cytosol. And leptin- and resistin- vesicles were not co-localized (Fig.3.1A and Fig.3.1.1). (However, at the Golgi apparatus place, there was extensive co-localization of leptin and resistin molecules, suggesting that they both are expressed and produced from the same localization, i.e., the Golgi-apparatus; Fig.3.1.2). Confocal co-localization studies were then performed on 3T3-L1 adipocytes overexpressed with GFP-tagged Rab5 (Rab5 is a molecular marker of early endosomes). As demonstrated in Fig.3.1B and C, neither leptin nor resistin molecules were accumulated in early endosomes. Glut-4 is the glucose transporter responsible for insulin-regulated glucose disposal in adipose tissue. Based on confocal imaging on adipocytes overexpressed with GFP-tagged Glut-4, it was found that both leptin and resistin were not carried in insulin-sensitive Glut-4 containing vesicles (Fig.3.1D and E). Plentiful vesicular storage makes it possible for acute release of these adipokines upon triggering, suggesting existence of the regulated secretory pathways. And distinct identities of leptin- and resistin- vesicles imply differences between the secretory routes of these adipokines.

Figure 3-1 Compartmentalization of leptin and resistin molecules in 3T3-L1 adipocytes revealed by confocal imaging. **[A]** Distribution of immunostained leptin (left, FITC conjugated secondary antibody) and resistin (middle, Atto 647 secondary antibody) molecules, and their co-localization (right, merge of left and middle). **[B]** Distribution of overexpressed Rab5-GFP (left) and immunostained leptin (middle, Atto 647 secondary antibody), and their co-localization (right). **[C]** Distribution of overexpressed Rab5-GFP (left) and immunostained resistin (middle, Atto 647 secondary antibody), and their co-localization (right). **[D]** Distribution of overexpressed Glut4-GFP (left) and immunostained leptin (middle, Atto 647 secondary antibody), and their co-localization (right). **[E]** Distribution of overexpressed Glut4-GFP (left) and immunostained resistin (middle, Atto 647 secondary antibody), and their co-localization (right). Scale bar = 10 μ m.

Figure 3.1.1 Distinct localization of leptin- and resistin- vesicles in 3T3-L1 adipocytes revealed by confocal imaging. **[A]** 3T3-L1 adipocytes overexpressed with leptin-EGFP were fixed, permeabilized, and immunostained with the rabbit anti-resistin primary antibody and the secondary antibody conjugated with Atto-647. **[B]** 3T3-L1 adipocytes overexpressed with resistin-EGFP were fixed, permeabilized, and immunostained with rabbit anti-leptin primary antibody and the secondary antibody conjugated with Atto-647. Scale bar = 10 μ m.

Figure 3.1.2 Co-localization of leptin and resistin molecules at the Golgi-apparatus place in 3T3-L1 adipocytes. Distribution of immunostained leptin (left, FITC conjugated secondary antibody) and resistin (middle, Atto 647 secondary antibody) molecules, and their co-localization (right, merge of left and middle) are shown accordingly. Scale bar = 10 μ m.

3.3 Trafficking of leptin- and resistin vesicles are differentially regulated

TIRFM, which selectively and evanescently illuminates the thin region (<200 nm) just above the interface between the glass coverslip and the adhered cell, is instrumental to provide insights on phenomena occurring at or near the plasma membrane. It complements the electrophysiological methods for study of exocytosis (Chen and Gillis, 2000). It has been revealed by TIRFM that secretory vesicles, such as large dense core secretory vesicles (LDCVs) in neuroendocrine cells and synaptic vesicles (SVs) in neurons undergo constant trafficking in the subplasmalemmal region (Holz and Axelrod, 2008). They move laterally while dwelling in the subplasmalemmal region. Such lateral movement can be characterized as random Brownian motion confined within a limited range by certain physical barrier or tethering forces (Zhang et al., 2008). Meanwhile, vesicles travel vertically, i.e., they arrive from the inner cytosol to the subplasmalemmal region and retrieve back after some time. The mobility and trafficking dynamics of secretory vesicles directly relate to their fusion competence and their secretion kinetics (Degtyar et al., 2007; Holz and Axelrod, 2008).

Subplasmalemmal leptin-vesicles (Fig.3.2A) and resistin-vesicles (Fig.3.2B) in live 3T3-L1 adipocytes were lightened by overexpressing EGFP-tagged leptin or resistin molecules and were individually resolved under TIRFM. Just like other secretory vesicles, these adipokine containing vesicles undertake constant lateral and vertical trafficking. The movement of individual vesicles was tracked at 0.5s time interval for 2 min to obtain information about the velocity of lateral movement and the dwell time of vesicles which emerged and then disappeared during imaging. Typical trajectories of lateral motion of a leptin vesicle (top) and a resistin vesicle are depicted in Fig.3.2.

Figure 3-2 Typical TIRFM images of 3T3-L1 adipocyte overexpressed with leptin-EGFP [A] or resistin-EGFP [B]. Individual leptin or resistin- containing vesicles can be resolved as bright dots. Typical motion trajectories of a subplasmalemmal leptin [A] and a resistin [B] vesicle are depicted on the right. The scale bars = 200 nm.

It has been shown that the cocktail of insulin and high concentration of glycolytic substrates (glucose and pyruvate) (abbreviated as GPI) can synergistically and potently stimulate leptin secretion (Levy and Stevens, 2001), possibly through triggered vesicular exocytosis (Roh et al., 2000). GPI stimulation mimics the hyperinsulinemic-hyperglycemic conditions associated with type-2 Diabetes. Interestingly, it is evident from Fig.3.3A (top) that GPI significantly increased the velocity of leptin vesicles (10 GPI treated cells: 411.7 +/- 12.2 nm/s vs. 9 control cells: 239.4 +/- 7.4 nm/s; $p < 0.001$). The competence of vesicle fusion requires sufficient vesicle mobility (Degtyar et al., 2007). It is, therefore, not surprising that GPI stimulates not only leptin secretion but also its vesicle trafficking. Consistently, the rate of vesicle arrival (vertical transport) was also enhanced by GPI (total of 50.5 +/- 4.5 vesicles arrived in 2 min vs. 22.9 +/- 1.6 vesicles, $p < 0.001$) (Fig.3.3A, middle). But it is noteworthy

that the total number of visible vesicles in the subplasmalemmal region remained stable, meaning that the increased vesicle arrival was balanced by vesicle release and/or increased vesicle retrieval. On the other hand, the dwell time of the vesicles in the subplasmalemmal region was largely reduced by GPI (13.3 +/- 0.9 s vs. 17.1 +/- 0.9 s, $p < 0.05$) (Fig.3.3A, bottom), presumably because of enhanced vertical trafficking. This may also be due in part to that, under stimulation, vesicles were released shortly after arriving in the subplasmalemmal region.

In contrast to leptin vesicles, the lateral and vertical trafficking of resistin vesicles was largely reduced by GPI (Fig.3.3B, top and middle), implying the inhibitory effect of GPI on vesicular release of resistin. Specifically, the velocity and arrival rate of resistin vesicles in 9 GPI treated cells were significantly reduced as compared to 9 control cells (78.1 +/- 3.0 nm/s vs. 197.5 +/- 8.7 nm/s, $p < 0.001$; and 21.1 +/- 2.8 vesicles arrived in 2 min vs. 29.7 +/- 1.5 vesicles, $p < 0.05$). And the dwell time of resistin vesicles was elongated by GPI treatment (20.2 +/- 0.7 s vs. 18.1 +/- 0.8 s, $p < 0.05$). Clearly, GPI differentially modulates the trafficking, thus likely secretion, of leptin- and resistin- vesicles.

Previous experiments have demonstrated that forskolin, which activates protein kinase A (PKA), inhibits insulin stimulated leptin secretion from primary adipocytes (Alonso-Vale et al., 2005). Consistently, we found that forskolin inhibited both the lateral and vertical trafficking of leptin vesicles, and increased the vesicle dwell time (Fig.3.3A). In addition, forskolin completely blocked the facilitating effects of GPI, suggesting that PKA may act downstream of GPI (Fig. 3.3A). Conversely, forskolin significantly enhanced the lateral and vertical trafficking of resistin vesicles, and reduced the vesicle dwell time (Fig.3.3B). And forskolin counteracted GPI inhibition on vesicle velocity and elongation on dwell time (Fig.3.3B). Taken together, our experiments showed that GPI and forskolin differentially regulate the trafficking (thus, likely exocytosis) of leptin- and resistin- vesicles.

Figure 3-3 Trafficking of leptin [A] and resistin [B] vesicles in 3T3-L1 adipocytes. Fluorescently labeled vesicles were individually tracked under TIRFM at 0.5s time interval for 2 min. The statistics (mean +/- SEM) of vesicle velocity (top), the number of vesicles arrived from inner cytosol (middle), the vesicle dwell time (bottom) was obtained in 3-4 independent experiments from 9 untreated leptin-expressing cells (Con), 10 GPI treated leptin-expressing cells (GPI), 9 GPI and forskolin (GPI + F) treated leptin-expressing cells, 9 forskolin treated leptin-expressing cells (F), 9 resistin treated leptin-expressing cells, 9 untreated resistin-expressing cells (Con), 9 GPI treated resistin-expressing cells (GPI), 9 GPI and forskolin treated resistin-expressing cells (GPI + F), 9 forskolin treated resistin-expressing cells (F), 8 leptin treated resistin-expressing cells. The number of vesicles analyzed for each condition is as indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ were determined by Student's t -test (for normally distributed arrived vesicle number), or Mann-Whitney test (for skewedly distributed vesicle velocity and dwell time).

Interestingly, leptin and resistin antagonize each other on vesicle trafficking. As compared to the control, applying resistin to the bath solution significantly slowed the lateral motion of leptin vesicles (9 cells: 136.3 ± 5.6 nm/s, $p < 0.001$ vs. control) and the rate of vesicle delivery (10.7 ± 3.6 vesicles arrived in 2 min, $p < 0.01$ vs. control), and increased the vesicle dwell time (21.1 ± 0.6 s, $p < 0.01$ vs. control) (Fig.3.3A). On the other hand, application of leptin to the bath solution significantly slowed the lateral motion of resistin vesicles (8 cells: 119.5 ± 4.3 nm/s, $p < 0.001$) and the rate of vesicle delivery (13 ± 1.6 vesicles arrived in 2 min, $p < 0.001$), and increased the vesicle dwell time (23.6 ± 0.8 s, $p < 0.01$) (Fig.3.3B). Therefore, it may be speculated that leptin and resistin inhibit the vesicular exocytosis of each other. Such interplay between leptin and resistin postulates the existence of autocrine regulation of adipokine secretion from adipocytes.

In addition, we occasionally observed full fusion of leptin-EGFP vesicles under GPI stimulation (Fig. 3.4A) and full fusion of resistin-EGFP vesicles under forskolin stimulation (Fig. 3.4B). As previously reported (Zhou et al., 2007), the fusion events can be characterized by (1) abrupt increase of fluorescence intensity about the vesicle center due to rapid release of fluorescent molecules, and (2) accompanying increase of fluorescent intensity in the peripheral region due to diffusion of released molecules. Thus, leptin-EGFP and resistin-EGFP are packed into fusion-competent vesicles. The secretion of leptin-EGFP and resistin-EGFP, without or with stimulation (GPI or forskolin), were also confirmed using western blot based on the antibody against EGFP. Secretion of EGFP from the 3T3-L1 adipocytes transfected with blank pEGFP-N1 vector could not be detected, suggesting that the EGFP tagged adipokines were likely released through the endogenous secretory pathways. The effects of GPI and forskolin on secretion of leptin-EGFP and resistin-EGFP (Fig.3.4) are consistent with their effects on trafficking of leptin- and resistin- vesicles (Fig.3.3), and with western blot results of endogenous leptin and resistin secretion (see section below). All these observations indicate that the overexpressed leptin-EGFP and resistin-EGFP are packed in the endogenous vesicles and regulated similarly as the endogenous proteins.

Figure 3-4 Leptin-EGFP and resistin-EGFP are packed into fusion-competent vesicles. [A and B] Sequential images of a leptin vesicle [A] or a resistin vesicle [B] labeled with EGFP in 3T3-L1 adipocytes undergoing a typical fusion event (upper), and their corresponding fluorescence changes over time (bottom) are shown. Times indicated are relative to the onset of fusion (indicated by the vertical dashed line). The solid lines and dotted lines represent the time courses of the mean fluorescence intensity in the inner circle and concentric annulus (Two concentric circles, ~1.0 μ m and ~1.3 μ m in diameter, Bai, et al. 2007). The scale bars represent 1 μ m. [C and D] Western blots of leptin-EGFP [C] and resistin-EGFP [D] secretion under control condition, or in presence of 10 μ M forskolin (F), GPI (1 mM pyruvate, 25 mM glucose, and 100 ng/ml insulin). 3T3-L1 adipocytes were transfected with leptin-EGFP or resistin-EGFP plasmids, 3-4 days before the experiments. Then the 3T3-L1 adipocyte culture was refreshed with the serum-free medium, and the medium with secreted leptin-EGFP protein and resistin-EGFP protein was collected 2 h later for western blot. Top panel shows the example western blots. The bottom is the statistics (mean $\pm$ SEM) from 3-4 independent experiments. The values are optical density of the blots in arbitrary unit. Student's t-test: ** p < 0.01, *** p < 0.001 vs. control.

3.4 Secretion of leptin and resistin is differentially regulated

Western blot was used to assay secretion of endogenous leptin and resistin from 3T3-L1 adipocytes. The top panel of Fig.3.5A (b) presents western blots of leptin (resistin) secretion from control cells or cells treated with different chemicals (GPI, forskolin, leptin, or resistin). The bottom panels of Fig.3.5A and B present the statistics (mean $\pm$ SEM) of the western blots quantified by optical density from 5-6 independent experiments. The western blot assays of secretion agree well with the observations on vesicle trafficking, i.e., all the treatments similarly affect vesicle trafficking and actual secretion. These corroborate the notions that leptin-EGFP and resistin-EGFP labeled vesicles are endogenous vesicles and trafficking of leptin- and resistin- vesicles are intimately related to the actual secretion of these adipokines.

Consistent with the previous study (Levy and Stevens, 2001), Fig.3.5A demonstrates that leptin secretion occurred in the absence of any stimulation and it was potently stimulated by the application of GPI. Therefore, it appears that leptin secretion is achieved through both constitutive and regulated secretory pathways. The constitutive secretion of leptin was greatly reduced by forskolin. In addition, GPI-stimulated secretion of leptin was completely abolished by forskolin, agreeing with the previous reported (Alonso-Vale et al., 2005) .

Resistin also undertakes both constitutive and regulated secretory pathways (Fig.3.5B). GPI and forskolin regulate resistin secretion in the ways opposite to their influences on leptin secretion. Specifically, it was observed that forskolin increased resistin secretion whereas GPI reduced it. Therefore, in line with our observations on vesicle trafficking, insulin/glycolytic substrates and PKA differentially regulate leptin and resistin secretion. The complete elimination of the GPI effects (its facilitation in leptin secretion and inhibition in resistin secretion) by forskolin suggests that PKA may act downstream of insulin in the regulated secretory pathways of both leptin and resistin. Also in agreement with the results on vesicle trafficking, leptin and resistin antagonized each other on secretion. In other words, application of resistin in the medium inhibited leptin secretion, and on the other hand, application of leptin suppressed resistin secretion (Fig.3.5).

Figure 3-5 Western blots of leptin [A] and resistin [B] secretion under control condition (Con), or in presence of 10 μ M forskolin (F), 10 μ M forskolin plus GPI (1 mM pyruvate, 25 mM glucose, and 100 ng/ml insulin) (GPI+F), 0.1 μ M leptin or resistin, GPI. The 3T3-L1 adipocyte culture was refreshed with the serum-free medium, and the medium with secreted leptin and resistin was collected 2 h later for western blot. Top panel shows the example western blots. The bottom is the statistics (mean $\pm$ SEM) from 5-6 independent experiments. The values are optical density of the blots in arbitrary unit. Student's t-test: ** $p < 0.01$, *** $p < 0.001$ vs. control.

3.5 Calcium dependence of leptin and resistin secretion

Regulated vesicular secretion is usually calcium dependent. A classic example is neurotransmitter release from synaptic vesicles in neurons. Increase of intracellular free Ca^{2+} by Ca^{2+} influx through voltage-dependent Ca^{2+} channels is required to drive fusion of the readily releasable vesicles with the plasma membrane, and to dock and prime vesicles into readily releasable state, by triggering rapid actions of secretory proteins (Burgoyne and Morgan, 2003). Although such Ca^{2+} triggering mechanism is obviously beneficial to ensure rapid neurotransmission, neuroendocrine cells also share the similar mechanism to release hormones from large dense core secretory vesicles into circulation (Burgoyne and Morgan, 2003). Are GPI stimulated leptin secretion and forskolin stimulated resistin secretion calcium dependent? To address this question, western blot of leptin and resistin secretion was performed, with or without depletion of extracellular Ca^{2+} , with or without depletion of intracellular Ca^{2+} , and with or without GPI or forskolin stimulation (Fig.3.6A - D). It was found that both basal and GPI stimulated leptin secretion were significantly suppressed when external calcium was depleted by calcium chelator EGTA or when internal calcium was depleted by membrane permeable calcium chelator BAPTA-AM. Therefore, both constitutive and regulated leptin secretions are Ca^{2+} dependent. In contrast, only basal, but not forskolin-stimulated, resistin secretion depends significantly on Ca^{2+} .

As expected from the observation that Ca^{2+} depletion did not inhibit forskolin-stimulated resistin secretion, forskolin did not trigger any Ca^{2+} signal based on Ca^{2+} photometry measurement (Fig.3.6E). But surprisingly, despite that GPI-stimulated leptin secretion requires Ca^{2+} , GPI was also not able to elicit any acute increase in cytosolic free Ca^{2+} . Similar observation was reported in primary rat adipocytes (Kelly et al., 1989). It implies that GPI stimulates leptin secretion not through acute increase in intracellular Ca^{2+} concentration. It is worth pointing out that Ca^{2+} imaging (Fig.3.6E) was undertaken in the time scale of min while the secretion of adipokines was measured in 2 h (Fig.3.5). Thus, the possibility that GPI and forskolin may modulate intracellular Ca^{2+} in a slow rate cannot be ruled out. Taken together,

external and internal calcium are important for both basal and regulated leptin secretion, and for basal resistin secretion.

Figure 3-6 Calcium dependence of leptin and resistin secretion in 3T3-L1 adipocytes. [A, B, C, D]. Example western blots (top) and statistics (bottom: mean $\pm$ SEM) from 3-4 independent experiments quantified by optical density of the blots of leptin [A and C] and resistin [B and D] secretion. In A and B, 3T3-L1 adipocyte culture was furnished with the fresh serum-free medium containing 1.8 mM Ca^{2+} , without (Con) or with 10 mM Ca^{2+} chelator EGTA (w. EGTA), or with stimulation (GPI or forskolin denoted as F), or with stimulation and with EGTA (GPI w. EGTA and F w. EGTA). In C and D, 3T3-L1 adipocytes were incubated without or with membrane permeable Ca^{2+} chelator BAPTA-AM (50 μ M) for 15 min (w. BAPTA) in the calcium-free bath solution. The cells were then washed and incubated with the fresh serum-free medium containing 1.8 mM Ca^{2+} , without stimulation (Con), or with stimulation (GPI or F). The medium with secreted leptin and resistin was collected 2 h later for western blot. Student's t-test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. Con. [E] Typical recordings of intracellular Ca^{2+} concentration from 3T3-L1 adipocyte in response to forskolin, GPI, calcimycin and high K^+ stimulation (from left to right). The Ca^{2+} concentration is reported by the ratio between the intensities of fluorescence emission when the Ca^{2+} sensitive dye (Fura2) is excited at 340 nm and 380 nm.

As a control experiment, application of calcium ionophore calcimycin resulted in sharp increase in $[Ca^{2+}]_i$ (Fig.3.6E). Interestingly, high K^+ solution which depolarizes the cell membrane also caused increase of $[Ca^{2+}]_i$, albeit with slow rise as compared to the responses in excitable cells such as neurons and neuroendocrine cells which have abundant voltage-gated Ca^{2+} channels. This observation may suggest the existence of slowly activated voltage-gated Ca^{2+} channels or Ca^{2+} carriers in 3T3-L1 adipocytes that may be involved in the calcium dependent secretion of adipokines.

3.6 Conclusion and Discussion

It has been well-documented that leptin and resistin are adipocyte-derived polypeptide hormones implicated critically in metabolic homeostasis (Rondinone, 2006; Rosen and Spiegelman, 2006). The functions and the secretory pathways of these adipokines, however, are still poorly understood. Particularly, very little is known about resistin. In the present work, we provide evidences that, in 3T3-L1 adipocytes, 1) leptin and resistin are compartmentalized into different vesicles. And these vesicles are distinct to early endosomes and Glut-4 containing vesicles; 2) In addition to constitutive secretion, both adipokines are released through regulated secretion, likely, regulated vesicular exocytosis, because all the reagents we tested affect their secretion and vesicle trafficking similarly; 3) The vesicle trafficking and secretion of leptin and resistin are differentially regulated by insulin/glycolytic substrates and protein kinase A; 4) Constitutive secretion of both leptin and resistin are Ca^{2+} dependent. And GPI-stimulated secretion of leptin, but not forskolin-stimulated secretion of resistin, is Ca^{2+} dependent, despite that GPI cannot induce acute increase of $[Ca^{2+}]_i$; and 5) Interplays exist between these two adipokines. Specifically, they are all subject to regulations by insulin/glycolytic substrates, PKA and Ca^{2+} ; and leptin and resistin antagonize each other on vesicle trafficking and secretion.

Studies have been carried out to identify the vesicular carriers responsible for the accumulation and transport of leptin. Using density and velocity gradient centrifugation (Roh

et al., 2001), immunofluorescence confocal microscopy (Barr et al., 1997; Roh et al., 2001), and electron microscopy based on immunolabeling (Bornstein et al., 2000), it has been revealed that leptin molecules aggregate into low-density vesicles whose molecular identity is unknown. And leptin containing vesicles are distinct from vesicles that contains Glut-4 (Barr et al., 1997), or lipoprotein lipase (Roh et al., 2001), or adiponectin (another adipokine) (Xie et al., 2008b). To our best knowledge, vesicular compartmentalization of resistin has not yet been reported.

Here, confocal imaging based on immunostaining and TIRFM imaging on live 3T3-L1 adipocytes overexpressed with fluorescent leptin or resistin revealed that these adipokines are packed into numerous vesicles. These vesicles are not identical to Glut-4 containing vesicles that are also sensitive to insulin and not identical to endosomes that often involve in the exocytotic pathways. It appears that these vesicles are similar to hormone containing secretory vesicles in endocrine cells such as chromaffin cells (Gasman et al., 1997; Steyer et al., 1997) and chromaffin-derived PC12 cells (Zhang et al., 2008) in terms of size and characteristics in trafficking. In addition, reagents that stimulate (or inhibit) the secretion of these adipokines also facilitate (or reduce) its vesicle trafficking. These observations support the view that these adipokines can be released through regulated vesicular exocytosis.

Whether adipokines are released by constitutive secretion or regulated secretion (exocytosis) or both is still a question under scrutiny. In constitutive pathways, secretory compounds are continuously secreted at a rate proportional to its synthesis without the need of stimulation, whereas, in regulated exocytotic pathways, proteins are stored into secretory vesicles and are discharged rapidly into extracellular space only in response to specific stimulations. Constitutive (basal) secretion of adipokines is presumably needed to set the basal metabolic activities. For example, basal leptin level is necessary to suppress appetite (or to avoid constant hunger). Our results show that both leptin and resistin are constitutively secreted. On the other hand, leptin and resistin also utilize regulated secretory pathways (vesicular exocytosis), because there are a large number of vesicular storage, and GPI or forskolin can acutely stimulate or inhibit secretion and vesicle trafficking of these adipokines (within 2h for

western blot experiments, and within 30 min for TIRFM imaging). Regulated secretion of adipokines is presumably important for acute response to metabolic conditions. For example, in response to hyperinsulinemia after a meal or other stimuli, leptin secretion is greatly enhanced as a signal of nutritional status to the hypothalamus thereby increasing insulin sensitivity, inhibiting food intake, enhancing energy expenditure and regulating body fat.

Co-immunostaining of endogenous leptin and resistin showed that these adipokines are accumulated in different types of vesicles. Their distinct identities were further confirmed by immunostaining of endogenous resistin in leptin-EGFP overexpressed cells and immunostaining of endogenous leptin in resistin-EGFP overexpressed cells (Fig.3.1.1). They different compartmentalization implies that they are secreted through distinct pathways. Indeed, we found that the secretion and vesicle trafficking of leptin and resistin are oppositely modulated by insulin/glycolytic substrates and PKA.

Figure 3-7 The effect of cycloheximide (CHX), a protein synthesis inhibitor, on intracellular leptin [A] and resistin [B]. Example western blots (top) and statistics (bottom: mean +/- SEM) from 3 independent experiments quantified by optical density of the blots of intracellular leptin [A] and resistin [B]. 3T3-L1 adipocyte culture was furnished with the fresh serum-free medium, without (Con) or with CHX 25 μ M (w. CHX), or with stimulation (GPI or forskolin denoted as F), or with stimulation and with CHX 25 μ M (GPI w. CHX and F w. CHX). After two hours, the cells were collected and lysed for western blot. The PVDF membrane was incubated with the primary antibodies for 4-6 h. Student's *t*-test: **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 vs. Con.

It has been shown that insulin upgrades leptin expression (Barr et al., 1997) and downgrades resistin expression (Haugen et al., 2001; Shojima et al., 2002; Chen et al., 2005). But the acute effects we observed are likely resulting from facilitation of regulated vesicular secretion of pre-stored adipokines instead of increased protein synthesis. Although GPI increased leptin expression and forskolin increased resistin expression in 3T3-L1 adipocytes (Fig.3.7), application of a protein synthesis inhibitor (cycloheximide), which completely eliminated GPI and forskolin induced protein synthesis (Fig.3.7), was not able to block the GPI-enhanced leptin secretion or forskolin-enhanced resistin secretion (Fig.3.8). In addition, cycloheximide could not block the effects of forskolin and resistin on leptin secretion or effects of GPI and leptin on resistin secretion (Fig.3.8). In agreement with our study, Bradley et al also showed that insulin stimulates leptin secretion from a preexisting intracellular pool in rat primary adipocytes (Bradley and Cheatham, 1999).

It is not surprising that the two adipokines are regulated differently and inhibit each other, considering that they counteract in some metabolic regulations such as insulin sensitivity and gluconeogenesis (Banerjee et al. 2004; Ogawa et al. 1999; Rossetti et al. 1997; Stepan et al. 2001a). The functional links between leptin and resistin have been revealed by several studies which showed that leptin treatment decreased resistin expression in ob/ob mice (Asensio et al. 2004; Rajala et al. 2004) and loss of resistin improved glucose homeostasis in leptin deficiency (Qi et al. 2006). Here, we show that leptin and resistin inhibit trafficking and secretion of each other at autocrine level. Intuitively, the autocrine regulations are important to the autoregulation of the fat mass and to balance the actions of various adipokines in response to complex metabolic signaling. Although leptin receptors in the hypothalamus is considered as the major mediator of leptin functions, leptin receptors have been found ubiquitously in most other tissues including adipose (Kielar et al. 1998). This suggests a universal, although not fully understood, role of leptin at autocrine, paracrine and endocrine levels. But the putative resistin receptors have not yet been identified.

Figure 3-8 Western blots showing the effect of cycloheximide (CHX) on leptin [A] and resistin [B] secretion. The 3T3-L1 adipocytes were incubated with CHX 25 μ M in the serum-free medium, under control condition (Con), or in presence of 10 μ M forskolin (F), 10 μ M forskolin plus GPI (1 mM pyruvate, 25 mM glucose, and 100 ng/ml insulin) (GPI+F), 0.1 μ M leptin or resistin, GPI. After two hours, the medium with secreted leptin and resistin were collected for western blot. The PVDF membrane was incubated with the primary antibodies for 18-22 h. Top panel shows the example western blots. The bottom is the statistics (mean $\pm$ SEM) from 3 independent experiments. The values are optical density of the blots in arbitrary unit. Student's *t*-test: **p* < 0.05, ***p* < 0.01 vs. control.

The role of calcium on leptin secretion has been studied previously in primary rat adipocytes (Levy et al., 2000; Cammisotto and Bukowiecki, 2004). In contrast to our observation in 3T3-L1 adipocytes, these studies found that basal leptin secretion is calcium independent. On the other hand, these studies and ours all indicated that insulin-stimulated leptin secretion is calcium dependent. However, we show that insulin is not able to elicit acute increase in $[Ca^{2+}]_i$, agreeing with the previous report (Kelly et al., 1989). This seems contradictory to the calcium dependence of GPI stimulated leptin secretion as observed in western blot and

trafficking of leptin vesicles. Such contradiction was also reported by Cammisotto et al in primary rat adipocytes (Arner et al., 1990; Cammisotto and Bukowiecki, 2004). These authors argued that external calcium is needed for insulin stimulated leptin secretion by enhancing glucose uptake although insulin does not affect extracellular calcium uptake. It is also conceivable that depletion of external calcium could gradually lead to a lower basal intracellular concentration, which, in turn, may affect glucose uptake, or directly affect the constitutive and regulated secretory pathways. It has been shown that depleting intracellular Ca^{2+} using BAPTA-AM diminishes >95% of insulin-stimulated glucose uptake by reducing the translocation as well as the final fusion steps of Glut4 (Whitehead et al., 2001). In comparison to leptin secretion, basal resistin secretion is also calcium dependent while forskolin-stimulated resistin secretion does not require calcium uptake. Clearly, calcium regulation on leptin and resistin secretion from adipocytes is markedly different from that on vesicular exocytosis in neurons and neuroendocrine cells.

In summary, we have comparatively studied several key aspects of leptin and resistin secretion, specifically, vesicle storage, vesicle trafficking, insulin/glycolytic substrates and PKA regulation. Further investigations are certainly required to reveal the commons, differences, and interplays between the secretory pathways of all adipokines in order to understand how they act synergistically or antagonizingly in response to complex metabolic signals.

3.7 Acknowledgement

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CHAPTER 4 THE CROSS-TALKS BETWEEN ADIPOKINES AND CATECHOLAMINES

4.1 Introduction

It has been increasingly recognized that adipose tissue, as the largest endocrine organ, play a pivotal role in regulating energy homeostasis via secretion of a variety of hormones called adipokines and via the intricate communication with other endocrine cells (Miner, 2004; Rosen and Spiegelman, 2006; Trujillo and Scherer, 2006; Lago et al., 2009). The functions and regulations of adipokines, and their interplays with other hormonal factors in the complex metabolic network are, however, still poorly established.

Among all adipokines, leptin and resistin have attracted much attention because of their critical involvement in energy metabolism and their relevance with metabolic disorders such as diabetes mellitus and obesity (Steppan et al., 2001a; Rondinone, 2006). These adipokines regulate various aspects of metabolism (e.g., lipolysis and gluconeogenesis) in collaboration with other metabolic hormones, notably insulin secreted from pancreatic β -cells (Steppan and Lazar, 2002; Rabe et al., 2008; Lago et al., 2009). Their direct interactions with insulin have been demonstrated in a number of studies. Specifically, insulin enhances leptin secretion whereas it inhibits resistin secretion (Barr et al., 1997; Pui et al., 2009; Ye et al., 2010). On the other hand, both leptin and resistin inhibit glucose-stimulated insulin secretion (Emilsson et al., 1997; Nakata et al., 2007). In addition to this well-recognized adipo-pancreatic (or adipoinsular) axis, the notion of adipo-adrenal axis is emerging (Glasow and Bornstein, 2000). Like leptin and resistin, catecholamines also stimulate lipolysis (Arner, 1999). Impairment of catecholamine-induced lipolysis in adipocytes is thought to contribute to the development of obesity (Jocken and Blaak, 2008). And catecholamines may directly regulate adipokine

secretion because adrenergic (catecholamine) receptors have been found on adipocytes (Fain and Garcia-Sainz, 1983; Scriba et al., 2000). On the other hand, it has been shown that leptin inhibits cortisol secretion from adrenocortical cells (Glasow et al., 1998) and stimulates catecholamine secretion from chromaffin cells in the adrenal medulla (Takekoshi et al., 1999).

In Chapter 3, we have comparatively studied several key aspects of leptin and resistin secretion, specifically, vesicle storage, vesicle trafficking, and insulin/glycolytic substrates regulation of these adipokines. Here in Chapter 4, using adipocytes differentiated from 3T3-L1 fibroblasts and PC12 cells derived from rat chromaffin cells as the in-vitro cell models, we sought to investigate the cross-talks between adipokines (leptin and resistin) and catecholamines (epinephrine, norepinephrine, and dopamine), i.e., how they regulate vesicle trafficking and secretion of each other. This study provides new insights of the adipo-adrenal axis in metabolism.

4.2 Catecholamines inhibit leptin and resistin secretion from 3T3-L1 adipocytes

Using western blot analysis, we examined the effects of catecholamines on the secretion of leptin and resistin from 3T3-L1 adipocytes. As shown in Fig.4.1, incubating the adipocytes with 1 μ M of epinephrine, norepinephrine, or dopamine for 2 hr significantly inhibited the secretion of both leptin and resistin. It indicates that all catecholamines, packed together in the large dense core vesicles (LDCVs) in chromaffin or PC12 cells, act collectively to inhibit secretion of the two adipokines.

Figure 4-1 Catecholamines inhibit leptin [A] and resistin [B] secretion from 3T3-L1 adipocytes. Top panel shows the representative immunoblots of leptin or resistin released to the culture medium in 2 hr, without (control or Con) or in presence of epinephrine (E, 1 μ M) or norepinephrine (NE, 1 μ M) or dopamine (DA, 1 μ M). GAPDH in the cell lysate serves as a loading control. The bottom panel shows the statistics (mean $\pm$ SEM, n = 4-5) of the optical density of the blots normalized to the density of GAPDH blots in arbitrary unit (a.u.). Student's *t*-test: ** *p* < 0.01, *** *p* < 0.001 vs. control.

Both α - and β -adrenergic receptors are expressed in white adipocytes (Fain and Garcia-Sainz, 1983). The suppressive effect of epinephrine (Fig.4.2A) and norepinephrine (not shown) on adipokine secretion was essentially relieved by co-application of propranolol (10 μ M, β -adrenergic receptor antagonist), but not affected by phentolamine (10 μ M, α -adrenergic receptor antagonist). The inhibitory effect of dopamine (Fig.4.2B) was also abolished by propranolol, but not by phentolamine or haloperidol (10 μ M, an antagonist for D₂, D₃, and D₄ dopaminergic receptors) or SCH-23390 (10 μ M, an antagonist for D₁ and D₅ dopaminergic receptors). Therefore, all catecholamines inhibit leptin and resistin secretion by activating β -adrenergic receptors on 3T3-L1 adipocytes.

4.3 Catecholamines reduce vesicle trafficking and secretion of leptin and resistin in 3T3-L1 adipocytes

In Chapter 3, we showed that leptin and resistin are segregated into distinct vesicles in 3T3-L1 adipocytes, and they can be selectively labeled by overexpressing leptin-EGFP or resistin-EGFP. In complement with electrophysiological and electrochemical measurement of vesicular secretion, total internal reflection fluorescence microscopy (TIRFM) is instrumental to reveal the trafficking and fusion of fluorescently labeled secretory vesicles in the subplasmalemmal region by selectively illuminating the thin membrane section (<200 nm) just above the interface between the coverslip and the cell membrane. Using TIRFM, what we showed in Chapter 3 is that, just like other secretory vesicles in neurons or endocrine cells (Holz and Axelrod, 2008), the trafficking dynamics of leptin- and resistin- vesicles is positively correlated with vesicle fusion competence and secretion kinetics. In other words, a reagent, which stimulates (or inhibits) leptin or resistin secretion, also stimulates (or inhibits) the lateral and vertical trafficking of leptin or resistin vesicles. So, it is conceivable that the inhibitory effect of catecholamines on secretion of leptin and resistin may be attributable to reduced vesicle trafficking of these adipokines.

Figure 4-3 Catecholamines reduced trafficking of leptin [A] and resistin [B] vesicles in 3T3-L1 adipocytes through the β -adrenergic receptors. Top panel presents a typical TIRFM image of 3T3-L1 adipocyte overexpressed with leptin-EGFP [A] or resistin-EGFP [B]. Each bright dot represents a leptin- or resistin- vesicle. The trajectory of the lateral movement of a leptin or a resistin vesicle in the subplasmalemmal region (scale bars = 200 nm) is depicted at the right. The middle and bottom panels show the statistics of the vesicle velocity and the rate of vesicle arrival to the subplasmalemmal region in 2-min imaging time, without (control or Con) or with 30 min incubation of epinephrine (E, 1 μ M) or norepinephrine (NE, 1 μ M) or dopamine (DA, 1 μ M) or epinephrine plus phentolamine (10 μ M, 30-min pretreatment) or epinephrine plus propranolol (10 μ M, 30-min pretreatment) or dopamine plus phentolamine (10 μ M, 30-min pretreatment) or dopamine plus propranolol (10 μ M, 30-min pretreatment). The values represent mean $\pm$ SEM (8 cells from 3-4 independent experiments and $\sim$ 500 vesicles in each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. epinephrine or dopamine treatment alone. Mann-Whitney test was used for skewedly distributed vesicle velocity. Student's t -test was used for normally distributed vesicle arrival rate.

Individual leptin-EGFP labeled vesicles (Fig.4.3A) and resistin-EGFP labeled vesicles (Fig.4.3B) in 3T3-L1 adipocytes were resolved under TIRFM and tracked for 2-min at 0.5 s time interval to obtain information about the average velocity of vesicle's lateral movement (parallel to the plasma membrane) in the subplasmalemmal region and the rate of vesicle arrival from the cytosol to the subplasmalemmal region (vertical trafficking). As evidenced from Fig.4.3, all catecholamines reduced both lateral and vertical trafficking of leptin and resistin vesicles.

The majority of newly arrived leptin or resistin vesicles fuse with the cell membrane, as indicated by transient increase of fluorescence intensity due to rapid discharge of fluorescent molecules followed by slightly delayed fluorescence increase in the peripheral region due to diffusional spread of released molecules and final disappearance of fluorescence (Fig.4.4).

Figure 4-4 Catecholamines decreased the fusion events of leptin- [A] and resistin- [B] vesicles in 3T3-L1 adipocytes through β -adrenergic receptors. Top panel presents the sequential images of a fusion event of a vesicle labeled by leptin-EGFP [A] or resistin-EGFP [A]. The scale bar = 1 μ m. The change of the mean fluorescence intensity over time within the region < 0.5 μ m away from the vesicle center (solid line) and the intensity changes within the annular region > 0.5 and < 0.65 μ m away from the vesicle center (dotted line) is shown in the middle panel. The bottom panel shows the average number of fusion events per cell within 2-min imaging time, without (control or Con) or with 30 min incubation of epinephrine (E, 1 μ M) or norepinephrine (NE, 1 μ M) or dopamine (DA, 1 μ M) or epinephrine plus phentolamine (10 μ M, 30-min pretreatment) or epinephrine plus propranolol (10 μ M, 30-min pretreatment) or dopamine plus phentolamine (10 μ M, 30-min pretreatment) or dopamine plus propranolol (10 μ M, 30-min pretreatment). The values represent mean $\pm$ SEM (8 cells from 3-4 independent experiments). Student's *t*-test: * p < 0.05, *** p < 0.001 vs. control. # p < 0.05, ### p < 0.001 vs. epinephrine or dopamine treatment alone.

Figure 4.4.1 Secretions of leptin-EGFP [A] and resistin-EGFP [B] from 3T3-L1 adipocytes were inhibited by epinephrine, norepinephrine, and dopamine. Top panel shows the representative western blots of leptin-EGFP or resistin-EGFP released to the culture medium in 2 hr, without (control or Con) or in presence of epinephrine (E, 1 μ M) or norepinephrine (NE, 1 μ M) or dopamine (DA, 1 μ M). The release leptin-EGFP or resistin-EGFP was not detected from non-transfected cells (Negative control). GAPDH serves as a loading control. The bottom panel shows the statistics (mean $\pm$ SEM, n = 3-4) of optical density of the blots normalized to the density of GAPDH blots in arbitrary unit (a.u.). Student's *t*-test: ** p < 0.01, *** p < 0.001 vs. control.

The frequency of the vesicle fusion events was significantly decreased by catecholamines. Propranolol, but not phentolamine, abolished the effects of catecholamines on vesicle trafficking and secretion, suggesting the involvement of β -adrenergic receptors (Fig.4.3 and

4.4). All these observations are expectedly consistent with the western blot analyses of actual adipokine secretion (Fig.4.1 and 4.2).

As we have demonstrated in Chapter 3, leptin-EGFP and resistin-EGFP are sorted in the endogenous vesicles, because secretion of leptin-EGFP and resistin-EGFP are identically regulated as endogenous leptin and resistin. Here, we show that secretion of leptin-EGFP and resistin-EGFP was also inhibited by catecholamines (Fig.4.4.1)

4.4 Leptin and resistin stimulate basal epinephrine secretion from PC12 cells

As epinephrine, norepinephrine, and dopamine are packed together in the LDCVs, the secretion of epinephrine was assayed using ELISA as the representative of catecholamine secretion. As shown in Fig.4.5, leptin (50 nM, 1 hr incubation) significantly enhanced epinephrine secretion from PC12 cells to 2 folds (to ~257.2%) as compared to control, while resistin (50 nM, 1 hr incubation) caused less, yet obvious, increase (to ~138.5%). It has been demonstrated that leptin promotes catecholamine synthesis in PKA (protein kinase A), PKC (protein kinase C), and MAPK kinase (mitogen-activated protein kinase kinase) dependent ways (Shibuya et al. 2002; Takekoshi et al. 1999; Takekoshi et al. 2001b). We used specific inhibitors to examine whether the stimulatory effects of leptin or resistin on catecholamine secretion are dependent on these kinases. As seen from Fig.4.5, in the presence of Ro32-0432 (2.5 μ M, PKC inhibitor) or H89 (10 μ M, PKA inhibitor) or PD98059 (50 μ M, MAPK kinase inhibitor), leptin or resistin induced epinephrine secretion was largely compromised or eliminated. These indicate that both leptin and resistin enhance basal catecholamine secretion from PC12 cells through PKA, PKC and MAPK kinase dependent pathways.

Figure 4-5 Leptin [A] and resistin [B] stimulate basal catecholamine secretion from PC12 cells. ELISA assay was used to quantify epinephrine secretion from PC12 cells in 1 hr, without (Control) or with 60-min incubation of leptin / resistin (50 nM) or leptin / resistin plus Ro32-0432 (2.5 μ M, 30-min pretreatment) or leptin / resistin plus H89 (10 μ M, 30-min pretreatment) or leptin / resistin plus PD98059 (50 μ M, 1 hr pretreatment) or leptin / resistin plus EDTA (0.5 mM, 10-min pretreatment) or leptin / resistin plus BAPTA-AM (30 μ M, 30-min pretreatment). Each data point represents mean $\pm$ SEM (n = 8-10). Student's *t*-test: * *p* < 0.05, *** *p* < 0.001 vs. control. # *p* < 0.05, ## *p* < 0.01, ### *p* < 0.001 vs. leptin or resistin treatment alone.

It has been reported that leptin stimulates catecholamine release from porcine chromaffin cells by inducing sustained rise of intracellular Ca^{2+} due to Ca^{2+} influx through voltage-dependent Ca^{2+} channels and internal Ca^{2+} release from endoplasmic reticulum (Takekoshi et al., 2001a). Here, we show that both leptin and resistin stimulated basal catecholamine secretion from PC12 cells depend on external and internal Ca^{2+} , because the adipokine stimulation was greatly reduced by addition of either EDTA (0.5 mM, membrane impermeable Ca^{2+} chelator) or BAPTA-AM (30 μM , membrane permeable Ca^{2+} chelator) (Fig. 4.5).

4.5 Leptin and resistin facilitate trafficking of LDCVs in PC12 cells

The lateral and vertical trafficking of catecholamine containing LDCVs (large dense core vesicles) in chromaffin cells or PC12 cells positively correlate with their vesicular secretion (Holz and Axelrod, 2008). Therefore, we speculated that leptin and resistin might promote the trafficking of LDCVs. LDCVs in PC12 cells were specifically labeled by overexpressing EGFP-tagged neuropeptide Y (NPY-EGFP) (Fig.4.6A). Indeed, as revealed by TIRFM, lateral as well as vertical trafficking of these vesicles was largely enhanced by 50 nM of leptin (Fig.4.6B) or resistin treatment (Fig.4.6C). Also in agreement with the ELISA results on actual epinephrine secretion (Fig.4.5), Ro32-0432 or H89 largely suppressed the vesicle trafficking and totally abolished the stimulatory effects induced by leptin and resistin, suggesting the involvement of PKC and PKA pathways. In contrast, PD98059 did not significantly affect vesicle trafficking without or with adipokine stimulation. Thus, MAPK kinase is likely not critically involved in the trafficking of LDCVs in PC12 cells.

Figure 4-6 Leptin [B] and resistin [C] promote the trafficking of catecholamines containing large dense core vesicles (LDCVs) in PC12 cells in PKC and PKA ways. [A] A typical TIRFM image of a PC12 cell overexpressed with NPY-EGFP (left). Each bright dot represents a LDCV. The trajectory of the lateral movement of a LDCV in the subplasmalemmal region is depicted at the right (scale bar = 200 nm). [B and C] The mean vesicle velocity and the vesicle arrival rate to the subplasmalemmal region in 2-min imaging time, after without (Control) or with 30-min incubation of leptin / resistin (50nM) or leptin / resistin plus Ro32-0432 (2.5 μM , 30-min pretreatment) or leptin / resistin plus H89 (10 μM , 30-min pretreatment) or leptin / resistin plus PD98059 (50 μM , 1 hr pretreatment). The values represent mean $\pm$ SEM (8 cells from 3-4 independent experiments and $\sim$ 400 vesicles in each group). ** $p < 0.01$, *** $p < 0.001$ vs. control. # $p < 0.05$, ### $p < 0.001$ vs. leptin or resistin treatment alone. Mann-Whitney test was used for skewedly distributed vesicle velocity. Student's t -test was used for normally distributed vesicle arrival rate.

4.6 Effects of leptin and resistin on triggered vesicle secretion of catecholamines

Figure 4-7 Leptin (but not resistin) enhances triggered exocytosis of catecholamines from PC12 cells. [A] Typical amperometric recordings in response to 2-min high K^+ stimulation without (control) or with 30-min pre-incubation of 50 nM leptin / resistin treatment [B] Total number of amperometric spikes normalized by the total responses before 30-min incubation of leptin / resistin or before 30-min waiting time (in control) on the same cell. Each data point shows mean $\pm$ SEM from 8 cells in each group. Student's t -test: ** $p < 0.01$ vs. control. [C] Photometry measurements of free intracellular Ca^{2+} in response to 2-min high K^+ stimulation without (control) or with 30-min pre-incubation of 50 nM leptin / resistin. The data is the average from 10 cells in each group. The Ca^{2+} concentration is indicated by ratio of fluorescence emission (F340 / F380) of Fura-2 at excitation wavelength of 340 nm and 380 nm.

Catecholamines can be acutely released via vesicular secretion upon Ca^{2+} triggering. Using single-cell amperometric measurements with a carbon-fiber-microelectrode (CFE) positioned on the cell surface, we investigated the effects of leptin and resistin on catecholamine secretion from PC12 cells triggered by high K^+ solution, which depolarizes the cell membrane and elicits Ca^{2+} influx. Fig.4.7A presents the representative amperometric traces in response to high K^+ stimulation from a PC12 cell without treatment and a cell treated with 50 nM leptin or 50 nM resistin for 30-min. Each amperometric current spike corresponds to single vesicle release, resulting from oxidization of catecholamine molecules on the voltage-biased CFM electrode (Xue et al., 2009). As demonstrated in Fig.4.7B, the total triggered secretion of catecholamines was largely augmented by leptin treatment. The leptin effect may be, in part, due to enhanced resting Ca^{2+} level and induced Ca^{2+} signal upon high K^+ stimulation (Fig.4.7C). In contrast to leptin, resistin did not increase the Ca^{2+} signal and triggered secretion despite its ability to facilitate basal catecholamine secretion (Fig.4.5).

4.7 Co-culturing of 3T3-L1 adipocytes and PC12 cells

To further investigate the interplays between adipokine and catecholamine secretion, we co-cultured 3T3-L1 adipocytes and PC12 cells using Trans-well co-culture system, in which the two types of cell culture are separated by a micro-porous membrane allowing exchange of soluble factors between the two cell types (Fig.4.8A). As shown in Fig.4.8B, one-day co-culturing caused decrease of leptin and resistin secretion from adipocytes and increase of epinephrine secretion from PC12 cells, in agreement with the observations in Fig.4.1 and 4.5.

Peculiarly, 3-4 days co-culturing leads to increase of leptin and resistin secretion and more significant increase of catecholamine secretion (Fig 4.9). These may be attributed to the observations that hypertrophy of adipocytes was promoted (as evidenced by more appearance of large oil droplets), and PC12 cells were differentiated into neuronal phenotype (as evidenced by the neurite growth) after 3-4 days co-culturing (Fig.4.9). The differences between short and long-term co-culturing imply the complex communications between the two

cell types via exchange of multiple signaling factors and the intricate long-term interactions between adipokines and catecholamines.

Figure 4-8 Co-culturing of 3T3-L1 adipocytes and PC12 cells. **[A]** Illustration of the Transwell co-culturing system. **[B]** Western blots of leptin and resistin secretion from 3T3-L1 adipocytes. Top panel shows the representative blots of leptin or resistin released to the culture medium in 2 hr after 24 hr of culturing alone (control or Con) or co-culturing with PC12 cells. GAPDH serves as a loading control. The lower panel shows the statistics (mean $\pm$ SEM, $n = 4-5$) of optical density of the blots normalized to the density of GAPDH blots in arbitrary unit (a.u.). **[C]** ELISA assays of epinephrine secretion from PC12 cells in 1 hr after 24 hr of culturing alone (control or Con) or co-culturing with 3T3-L1 adipocytes. The statistics (mean $\pm$ SEM) was obtained from 3-4 experiments. Student's t -test: *** $p < 0.001$ vs. respective control.

Figure 4-9 Co-culturing of 3T3-L1 adipocytes and PC12 cells for 3-4 days. **[A]** Leptin and resistin secretion from 3T3-L1 adipocytes were increased by co-culturing as revealed by western blot analysis. Top panel shows the representative blots of leptin or resistin released to the culture medium in 2 hr after 3-4 days of culturing alone (control or Con) or co-culturing with PC12 cells. GAPDH serves as a loading control. The bottom panel shows the statistics (mean $\pm$ SEM, $n = 3-4$) of optical density of the blots normalized to the density of GAPDH blots in arbitrary unit (a.u.). Student's t -test: *** $p < 0.001$ vs. control. **[B]** Epinephrine secretion from PC12 cells was significantly enhanced by co-culturing as revealed by ELISA measurements. The statistics (mean $\pm$ SEM) was obtained from 3-4 separate experiments. Student's t -test: *** $p < 0.001$ vs. control. **[C]** 3-4 day's co-culturing leads to hypertrophy of adipocytes (more appearance of large oil droplets) and neurite outgrowth of PC12 cells.

4.8 Conclusion and Discussion

Adipocytes play an essential role in metabolism and energy homeostasis through communication with other endocrine cells in the complex metabolic networks (Miner, 2004; Trujillo and Scherer, 2006). In addition to endocrine communication via blood circulation, the cross-talks can also occur at paracrine level as adipose tissue exists in many organs including pancreas and adrenal gland (Ehrhart-Bornstein et al., 2003; Pinnick et al., 2008).

The cross-talks between adipose tissue and the adrenal gland have been supported by the observations that aldosterone produced by adrenal cortex induces adipogenesis while adipocytes release some unidentified factors to stimulate aldosterone production (Ronconi et al., 2008). In addition, it has been shown that leptin stimulates catecholamine secretion from porcine chromaffin cells (Takekoshi et al., 1999). Here, we provide further evidence of the communications between adipocytes and chromaffin cells in the adrenal medulla (Fig.4.10). We show that leptin and resistin promote vesicle trafficking and secretion of catecholamines from rat chromaffin cell derived PC12 cells while catecholamines inhibit the vesicle trafficking and secretion of these adipokines from adipocytes differentiated from 3T3-L1 mouse embryonic fibroblast. Our study stresses the importance of the adipo-adrenal axis in metabolism. This notion is in line with the findings that adrenergic receptors are expressed on adipocytes (Fain and Garcia-Sainz, 1983) and leptin receptors are expressed on chromaffin cells (Glasow et al., 1998; Yanagihara et al., 2000). However, it is noteworthy that, in human, resistin is secreted abundantly by immune and epithelial cells (Steppan et al., 2001b; Patel et al., 2003). Although it was reported that resistin is secreted from human subcutaneous adipocytes (McTernan et al., 2002; Degawa-Yamauchi et al., 2003), other studies have demonstrated that resistin is highly expressed in human pre-adipocytes instead of mature human adipocytes (Savage et al., 2001; Janke et al., 2002b).

White adipocytes express both α - and β - adrenergic receptors (Fain and Garcia-Sainz, 1983). It has been reported that β -adrenergic agonist (isoproterenol) promotes expression and secretion of IL-6 and TNF- α from human abdominal adipose tissue and

3T3-L1 adipocytes, whereas it inhibits expression and secretion of adiponectin from mouse adipose explants and 3T3-L1 adipocytes (Fasshauer and Paschke, 2003; Fu et al., 2007a; Lago et al., 2009). Here, we show that all catecholamines synergistically inhibit leptin and resistin secretion through β -adrenergic receptors. Although evidence of dopaminergic receptor expression on adipocytes is absent, it is known that dopamine is able to stimulate adrenergic receptors (Clark et al., 1988; Lee et al., 1998). In agreement with our results, the previous studies have demonstrated that isoproterenol reduce leptin release from human adipocytes (Scriba et al., 2000), and blood infusion of isoproterenol results in decreased plasma level of leptin in human (Donahoo et al., 1997b). And norepinephrine, isoproterenol and CL-316243 (potent β_3 -adrenergic receptor agonist) inhibit insulin-stimulated leptin release from rat white adipocytes (Gettys et al., 1996; Cammisotto and Bukowiecki, 2002). We further demonstrate that secretion of resistin, which is packed into vesicles distinct to leptin vesicles (Ye et al., 2010) , is also inhibited by catecholamines. The inhibition of leptin and resistin secretion may be partly attributable to the reduced adipokine expression because isoproterenol inhibits leptin and resistin mRNA expression through Gs-protein coupled pathway in 3T3-L1 adipocytes (Kosaki et al., 1996; Fasshauer et al., 2001c). Such inhibition may also be accounted by the reduced vesicle trafficking (Fig.4.3).

Figure 4-10 Illustration of cross-talks between adipokines and catecholamines at endocrine or paracrine level.

Figure 4-11 Acetylcholine (Ach) stimulates secretion and vesicle trafficking of leptin and resistin from 3T3-L1 adipocytes via the muscarinic cholinergic receptor. [A and B] Top panel shows the representative western blots of leptin or resistin released to the culture medium in 2 hr, without (Con or control) or in the presence of acetylcholine (Ach, 1 μM) or acetylcholine plus atropine (antagonist for muscarinic cholinergic receptor; 10 μM , 30-min pretreatment) or acetylcholine plus tubocurarine (antagonist for nicotinic cholinergic receptor; 10 μM , 30-min pretreatment) or nicotine (agonist for nicotinic cholinergic receptor; 10 μM). GAPDH serves as a loading control. The bottom panel shows the statistics (mean $\pm$ SEM, $n = 3-4$) of optical

density of the blots normalized to the density of GAPDH blots in arbitrary unit (a.u.). **[C and D]** The statistics (mean $\pm$ SEM) of vesicle lateral velocity and vesicle arrival obtained from TIRFM experiments (8 cells from 3-4 experiments and ~500 - 600 vesicles in each group). ** $p < 0.01$, *** $p < 0.001$ vs. control. Mann-Whitney test was used for skewedly distributed vesicle velocity. Student's t -test was used for western blots and normally distributed vesicle arrival rate.

In addition to adrenergic receptors, adipocytes express other receptors as well including insulin receptors (Jarett et al., 1980) and acetylcholine receptors (Liu et al., 2004; Stephens et al., 2009), suggesting that adipokine secretion is highly regulated. Acetylcholine (ACh) is released from adrenal medulla and stimulates catecholamine secretion (Mizobe and Livett, 1983; Hirokami et al., 1994). On the contrary to the effect of catecholamines, acetylcholine suppresses lipolysis (Yang et al., 2009). We found that, in contrast to catecholamines, acetylcholine enhances secretion and vesicle trafficking of leptin and resistin from 3T3-L1 adipocytes through the muscarinic cholinergic receptors (Fig.4.11). This adds another dimension in the adipo-adrenal axis.

Although leptin and resistin are differentially regulated and sometimes function oppositely (Pui et al., 2009), they all stimulate basal catecholamine secretion in PKC, PKA and MAPK kinase dependent manner. This may be achieved through enhanced synthesis (Takekoshi et al., 2001b; Utsunomiya et al., 2001; Shibuya et al., 2002) and increased vesicle trafficking (Fig. 4.6). But only leptin (not resistin) significantly increases triggered secretion of catecholamines. This could be attributed to the leptin-enhanced vesicle trafficking and Ca^{2+} signaling (Fig.4.6 and 4.7C). In addition, leptin increases cAMP level as well as PKC activity and expression in porcine chromaffin cells (Takekoshi et al., 1999; Takekoshi et al., 2001b). It is well known that PKC and cAMP/PKA are potent promoters for triggered catecholamine secretion (Tachikawa et al., 1995; Yang et al., 2002b).

As both adipocytes and chromaffin cells secrete a variety of hormones, the communications between adipocytes and chromaffin cells shall be more complex than being uncovered here. This is evidenced by experiments of co-culturing 3T3-L1 adipocytes and PC12 cells. Long-term co-culturing (3-4 days) led to neurite outgrowth of PC12 cells possibly

due to neurotrophic effects of nerve growth factor (NGF) (Peeraully et al., 2004) and angiopoietin-1 (Kosacka et al., 2006) secreted by adipocytes. And conversely to the short-term co-culturing (1 day), long-term co-culturing caused increase of leptin and resistin secretion from 3T3-L1 adipocytes (Fig.4.9). This could be due to down-regulation of adrenergic receptors (Bottner et al., 1999) and promoted hypertrophy of adipocytes (Fig.4.9). And neuropeptide Y (NPY) secreted by chromaffin and PC12 cells are known to stimulate adipogenesis as well as increase the secretion of leptin and resistin (Kuo et al., 2007).

Both adipokines and catecholamines are critically involved in metabolism. These hormones act oppositely in some cases and synergistically in other cases. This study highlights the importance of adipo-adrenal axis and the intricate interactions between the metabolism-relevant hormones which should only be understood in spatially and temporally defined physiological contexts.

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CHAPTER 5 APELIN SECRETION AND EXPRESSION OF APELIN RECEPTORS IN 3T3-L1 ADIPOCYTES ARE DIFFERENTIALLY REGULATED BY ANGIOTENSIN TYPE 1 AND TYPE 2 RECEPTORS

5.1 Introduction

It is becoming increasingly clear that adipocytes, previously thought as the mere energy storage sites, play pivotal roles in regulating energy metabolism through secretion of a variety of signaling molecules called adipokines (Fruhbeck et al., 2001; Lago et al., 2009). As we have shown in Chapter 3 and 4, secretion of adipokines (leptin, resistin, etc) is highly regulated, and hence they are implicated with metabolic disorders, such as, obesity and diabetes mellitus (Matsuzawa, 2007; Ouchi et al., 2011; Than et al., 2011a).

Apelin, which has been known to be secreted by several cell types (e.g., neurons (Reaux et al., 2001), cardiomyocytes (Chen et al., 2003), endothelial cells (Kleinz and Davenport, 2004), is identified as a new member of the adipokine family (Boucher et al., 2005; Wei et al., 2005). Apelin is involved in various physiological functions, including cardiac muscle contraction (Ashley et al., 2005), vasodilatation (Jia et al., 2007), feeding control (Valle et al., 2008), and metabolic homeostasis (Higuchi et al., 2007; Dray et al., 2008; Attane et al., 2010). Its plasma level is elevated in obesity (Boucher et al., 2005). As apelin receptors are expressed in adipocytes (Wei et al., 2005; Castan-Laurell et al., 2008), it is not surprising that apelin exerts multifaceted influence on adipose tissue. It increases glucose uptake in adipocytes by enhancing insulin sensitivity (Dray et al., 2008; Yue et al., 2010); regulates secretion of other adipokines (Higuchi et al., 2007); inhibits adipocyte lipolysis (Yue et al., 2011); and promotes angiogenesis in adipose tissue (Kunduzova et al., 2008; Rayalam et al., 2008) and therefore likely adipose tissue growth (Rupnick et al., 2002).

Angiotensin II (AngII) is a peptide hormone, well-known for its regulations on blood vessel contraction and angiogenesis (Lucius et al., 1999). In adipose tissue, both white adipocytes and vascular endothelial cells produce AngII (Schling and Loffler, 2002; Engeli et al., 2003; Hung et al., 2010). The plasma level of AngII increases significantly in obesity (Goossens et al., 2003). In adipocytes, AngII has anti-lipolytic as well as lipogenesis effects (Jones et al., 1997a; Goossens et al., 2007) and potentiates insulin-stimulated glucose uptake (Juan et al., 2005). It acts through angiotensin type 1 receptor (AT₁) and type 2 receptor (AT₂) (Dinh et al., 2001). Both receptors are expressed in adipocytes (Mallow et al., 2000; Schling, 2002; Juan et al., 2005). Activation of the two types of receptor triggers distinct signaling pathways and usually produces antagonizing effects (Dinh et al., 2001; Johren et al., 2004). For examples, AT₁ signaling stimulates angiogenesis and adipogenesis while AT₂ signaling inhibits them (Chung et al., 1996; Di Filippo et al., 2005; Matsushita et al., 2006; Sarzani et al., 2008). Evidences have been demonstrated that AngII modulates secretion of several adipokines, including leptin, interleukin 6 (IL-6), IL-8, and plasminogen activator inhibitor-1 (PAI-1) (Skurk et al., 2001; Kim et al., 2002; Skurk et al., 2004). A recent study showed that inhibition of AngII synthesis or AngII receptors during adipogenesis of 3T3-L1 cells increases apelin expression and secretion (Hung et al., 2010), suggesting the regulatory roles of AngII on apelin secretion.

Here in Chapter 5, we investigated the effects of AngII on apelin secretion and APJ receptor (apelin receptor) expression in 3T3-L1 adipocytes and elucidated the distinct signaling pathways mediated by AT₁ and AT₂ receptors, respectively.

5.2 Effects of AngII on apelin secretion and expression in 3T3-L1 adipocyte

To reveal the effect of AngII and its dose and time dependence, apelin secretion was measured using enzyme immunoassays (EIA) after the 3T3-L1 adipocytes were incubated with AngII of various concentrations (1 nM – 10 μ M) and for different durations. As shown in Fig.5.1A, apelin secretion was acutely stimulated (within 30 min) by the high-doses of AngII

(1 or 10 μ M). Interestingly, biphasic dose-dependent response was observed for a longer period (2 or 24 hr) of AngII treatment, i.e., the low concentrations of AngII (1 or 10 nM) inhibited apelin secretion whereas the high concentrations of AngII (1 or 10 μ M) stimulated it (Fig.5.1B and 1C).

As reported previously, 77- amino acid apelin (preproapelin, ~8 kDa) can be secreted and detected in western blots as the indicator of apelin secretion (Xie et al., 2006), while its dimer (~16 kDa) is the major form of apelin found in tissues and cells (Lee et al., 2005a; Alastalo et al., 2011). In our experiments on 3T3-L1 adipocytes, perhaps surprisingly, secretion of apelin dimer can also be detected. To verify, we show that secretion of both preproapelin and its dimer were sensitive to siRNA inhibition on apelin expression (Fig.5.1.1). Secretion of the dimer forms of other adipokines has also been observed in human adipocytes (Simons et al., 2007). Using preproapelin as the indicator, the biphasic dose response of apelin secretion to long-term AngII stimulation (2 hr) was confirmed by western blot analyses (Fig. 5.1D). Such biphasic response was also observed for the intracellular expression of apelin dimer (Fig.5.1E). As a reference experiment, we also examined the effect of insulin. In agreement with the previous report (Boucher et al., 2005), 100 nM insulin significantly increased apelin expression and release from 3T3-L1 adipocytes.

The observed peculiar biphasic regulation of AngII on apelin secretion and expression clearly suggest the co-existence of stimulatory and inhibitory pathways. And from the results in Fig.5.1, it appears that the stimulatory pathway is faster and requires higher dose of stimulation as compared with the inhibitory pathway. In all the subsequent experiments, the medium dose of 100 nM AngII was used. And in all the subsequent western blot analyses, preproapelin and its dimer were used respectively as the indicators for extracellular secretion and intracellular expression of apelin.

Figure 5-1 Dose-dependent effect of AngII on apelin secretion and expression in 3T3-L1 adipocytes. [A-C] Enzyme immunoassay (EIA) measurement of apelin released to the medium after the adipocytes were treated without (Con as control) or with various concentrations of AngII (1 nM – 10 μM) or insulin (Ins, 100 nM) for different durations (30 min, 2 hr or 24 hr). Results are expressed as mean ± SEM (n = 6) from 3 - 4 independent culture samples. [D] Top panel shows the representative immunoblots of preproapelin (~ 8 kDa) released to the media in 2 hr and intracellular GAPDH (~37kDa) in cell lysates, under indicated conditions. The lower panel shows the statistics (mean ± SEM, n = 6) of optical density of the blots in arbitrary units (a.u.) normalized by optical density of the GAPDH blots. GAPDH, a constantly expressed intracellular protein, was used as the internal control. [E] Top panel shows the representative immunoblots of intracellular apelin dimer (~ 16 kDa) and GAPDH, after 8 or 24 hr incubation under indicated conditions. The lower panel shows the statistics (mean ± SEM, n = 5) of optical density of the apelin blots (a.u.) normalized by optical density of the GAPDH blots. Student's *t*-test: * *p* < 0.05, ** *p* < 0.01 vs. control.

Figure 5.1.1 Western blot detection of preproapelin and its dimer released and expressed in 3T3-L1 adipocytes. (on day 6 – 7). Gene silencing of apelin was used to block the apelin expression in adipocytes using Apelin-siRNA (Apelin siRNA (m): sc-44742, Santa Cruz Biotech). 77- amino acid preproapelin (~8kDa) was detected both in cell media and lysates while the majority form of apelin protein in cell lysates was the dimer (~16 kDa).

5.3 Angiotensin receptor type 1 (AT₁) and type 2 (AT₂) differentially regulate apelin secretion

Both AT₁ and AT₂ receptors are expressed in animal and human white adipocytes (Engeli et al., 1999; Schling, 2002; Juan et al., 2005) as well as in 3T3-L1 adipocytes (Mallow et al., 2000; Weiland and Verspohl, 2008). To differentiate the effects of AT₁ and AT₂ on the regulation of apelin secretion, we used specific antagonists to silence one particular receptor type and isolate the effect of the other type (Takekoshi et al., 2002). Specifically, activation of AT₁ receptor was inhibited by ZD7155 (1 μ M) (Kramer et al., 1998) and activation of AT₂ receptor was inhibited by PD123319 (1 μ M) (Blankley et al., 1991).

As demonstrated in Fig.5.2A, apelin secretion was acutely stimulated (30 min) by 100 nM AngII through the AT₁ receptors, while activation of AT₂ receptors had no effect. Under long-term stimulation (2 or 24 hr), the stimulatory effect of AT₁ receptors preserved while the inhibitory effect of AT₂ receptors became evident (Fig. 5.2B and C). This was confirmed by the western blot analyses (Fig. 5.2D). In the absence of AngII, inhibition of AT₁ or AT₂ receptors alone did not obviously affect the basal apelin secretion (Fig.5.2D), suggesting that these pharmacological compounds themselves do not interfere with apelin secretion. These results together with those in Fig.5.1 imply that low-dose of AngII inhibits apelin secretion through AT₂ signaling pathways and high-dose of AngII stimulates apelin secretion through AT₁ signaling pathways in a more acute manner. This is corroborated by the observations that, at a low-dose of AngII (10 nM), the stimulatory effect of AT₁ receptors was absent whereas the inhibitory effect of AT₂ receptors preserved (Fig.5.2E).

Figure 5-2 Apelin secretion in 3T3-L1 adipocytes is differentially regulated by angiotensin receptor type 1 and type 2 (AT₁ and AT₂) in presence of 100 nM AngII. **[A-C]** Enzyme immunoassay (EIA) measurement of apelin released to the medium after the adipocytes were treated without (Con as control) or with 100 nM AngII plus 1 μ M PD123319 (AT₂ receptor antagonist), or AngII plus 1 μ M ZD7155 (AT₁ receptor antagonist) for different durations (30 min, 2 hr or 24 hr). Results are expressed as mean $\pm$ SEM (n = 6) from 3 - 4 independent culture samples. **[D]** Top panel shows the representative immunoblots of preproapelin released to the media in 2 hr and intracellular GAPDH in cell lysates, under indicated conditions. The lower panel shows the statistics (mean $\pm$ SEM, n = 6) of optical density of the blots in arbitrary units (a.u.) normalized by optical density of the GAPDH blots. **[E]** EIA measurement of apelin released to the medium after the adipocytes were incubated for 2 hr under the indicated conditions. The results are shown as mean $\pm$ SEM (n = 6) of 3 - 4 independent samples. Student's t-test: * p < 0.05, ** p < 0.01 vs. control.

5.4 AT₂ mediated inhibition is cAMP and cGMP dependent

It has been shown in several cell types that activation of AT₂ receptors decreases the intracellular cAMP (Zhang and Pratt, 1996; Hansen et al., 2000) and cGMP levels (Sumners et al., 1991; Takekoshi et al., 2002). Therefore, we investigated the cAMP and cGMP dependence on AT₂ mediated inhibition on apelin secretion from 3T3-L1 adipocytes.

We used forskolin (cell-permeable adenylyl cyclase activator) to increase cAMP production and 8-bromo-cGMP (a membrane permeable cGMP analog) to increase intracellular cGMP level in adipocytes. As shown in Fig.5.3A, forskolin (10 μ M) enhanced apelin release from 3T3-L1 adipocytes, consistent with the previous finding (Mazzucotelli et al., 2008). The same treatment promoted apelin expression (Fig. 5.3B). In addition, Fig.5.3A and B indicate that the inhibitory effect on apelin secretion and expression induced by AT₂ activation was relieved by forskolin treatment. Similarly, application of 8-bromo-cGMP (100 μ M) also increased the basal apelin secretion and expression, and relieved the influence of AT₂ activation (Fig.5.3C and 3D). These results suggest that AT₂ activation inhibits apelin secretion, at least in part, by reducing cAMP and cGMP levels. In contrast, increasing cAMP or cGMP level did not appreciably inhibit or potentiate the stimulating effect of AT₁ activation on apelin secretion.

Figure 5-3 Inhibitory effect of AT₂ activation on apelin secretion and expression is cAMP [A and B] and cGMP [C and D] dependent, as revealed by EIA measurements (A and C) and western blot analyses (B and D). Adipocytes were incubated for 2 hr (A and C) or 24 hr (B and D) under the indicated conditions. The chemical concentrations are: forskolin (10 μ M), 8-Bromo-cGMP (100 μ M), AngII (100 nM), PD123319 (1 μ M), ZD7155 (1 μ M). The results are shown as mean $\pm$ SEM (n = 4-6) of 3 – 4 independent samples. Student's t-test: * p < 0.05, ** p < 0.01 vs. control. # p < 0.05, ## p < 0.01 vs. AngII plus PD123319 treatment.

5.5 AT₁ mediated stimulation is PKC and Ca²⁺ dependent

It has been demonstrated that activation of protein kinase C (PKC) up-regulates apelin expression and inhibition of PKC blocks the insulin-induced apelin expression in adipocytes (Boucher et al., 2005). In line with this previous report, we found that 100 nM phorbol-12-myristate-13-acetate (PMA, a potent PKC activator) largely increased apelin secretion from 3T3-L1 adipocytes (Fig.5.4A). On the other hand, inhibition of PKC activities by Ro-32-0432 (a potent and selective inhibitor of PKC) did not affect apelin secretion, indicating that the basal PKC activities are not essential to the basal apelin secretion. But as shown in Fig.5.4A, Ro-32-0432 was able to eliminate the stimulatory effect of AT₁ activation on apelin secretion. Thus, it is likely that AT₁ signaling acts through activation of PKC. In comparison, AT₂ signaling seems to be PKC independent because Ro-32-0432 did not significantly affect the inhibitory effect of AT₂ activation.

Since Ca²⁺ is a co-factor for PKC activation, we investigated the Ca²⁺ dependence in the AT₁ signaling. As shown in Fig.5.4B, depleting external Ca²⁺ (2 mM) in the culture medium by addition of 10 mM EGTA affected neither the basal apelin secretion nor the AT₁ activation enhanced secretion. In contrast, depleting internal Ca²⁺ using a membrane permeable Ca²⁺ chelator (BAPTA-AM, 30 μ M) reduced the basal secretion and abolished the AT₁ activation enhanced secretion. Therefore, it may be concluded that AT₁ activation facilitates apelin secretion through triggering internal release of Ca²⁺ which, in turn, activates PKC. As shown in Fig.5.4C, activation of AT₁ by AngII can indeed elicit an obvious gradual increase of intracellular Ca²⁺ in the absence of external Ca²⁺.

Figure 5-4 Stimulatory effect of AT₁ activation on apelin secretion is PKC and Ca²⁺ dependent. [A and B] EIA measurement of apelin release after the adipocytes were treated (2 hr) without (Con as control) or with PMA (100 nM), or Ro32-0432 (2.5 μ M, 30 min pretreatment), or AngII (100 nM) plus PD123319 (1 μ M), or AngII plus PD123319 and Ro32-0432, or AngII (100 nM) plus ZD7155 (1 μ M), or AngII plus ZD7155 and Ro32-0432, or EGTA (10 mM), or AngII plus PD123319 and EGTA, or BAPTA-AM (30 μ M), or AngII plus PD123319 and BAPTA-AM. The results are shown as mean $\pm$ SEM (n = 6) of 3 independent samples. Student's t-test: * p < 0.05, ** p < 0.01 vs. control. # p < 0.05 vs. AngII plus PD123319 treatment. [C] Typical photometry recordings of free intracellular Ca²⁺ levels in 3T3-L1 adipocytes, without (control) or with stimulation of 100 nM AngII (30-min pre-incubation of 1 μ M PD123319).

5.6 Involvement of MAPK/ERK pathway

Evidences have shown in a number of cell types that AT₁ activation enhances MAPK/ERK signaling pathway while AT₂ activation suppresses it (Griendling et al., 1996; Dinh et al., 2001). And Boucher et al demonstrated that PD98059, a specific inhibitor of mitogen-activated protein kinase kinase (MAPK kinase), blocked the insulin-induced apelin expression in adipocytes (Boucher et al., 2005). So, it is conceivable that MAPK/ERK pathway promotes apelin expression and hence its secretion, and both AT₁ and AT₂ modulate apelin secretion through this pathway. As shown in Fig.5.5A and B, inhibition of MAPK kinase by 50 μ M PD98059 eliminated AT₁ activation induced apelin secretion and expression while it did not affect the basal levels. Obviously, MAPK/ERK pathway is indeed critical to AT₁ mediated enhancement. This is further supported by the observation that AT₁ activation significantly increased phosphorylation of ERK 1/2, the substrate of MAPK kinase (Fig.5.5B).

On the other hand, inhibition of MAPK kinases by PD98059 did not further potentiate the inhibition of apelin secretion by AT₂ activation (Fig.5.5A). Studies on other cell types have shown that AT₂ activation decreases ERK 1/2 phosphorylation by stimulating various phosphatases (Griendling et al., 1996; Dinh et al., 2001). In 3T3-L1 cells, we found that stimulation of AT₂ only caused marginal decrease of ERK 1/2 phosphorylation (Fig.5.5B). Taken together, it is likely that AT₂ mediated regulations are mainly achieved through cAMP and cGMP pathways (Fig.5.3) instead of MAPK/ERK pathway.

Figure 5-5 AT₁ and AT₂ receptors act through MAPK/ERK pathway. **[A]** EIA measurement of apelin release after the adipocytes were treated (2 hr) without (Con as control) or with MAPK kinase inhibitor (PD98059, 50 μ M, 1 hr pretreatment), or AngII (100 nM) plus PD123319 (1 μ M), or AngII plus PD123319 and PD98059, or AngII plus ZD7155 (1 μ M), or AngII plus ZD7155 and PD98059. **[B]** Western blot analyses of intracellular apelin expression (24 hr) under indicated conditions (same as in A). The results in A and B are shown as mean $\pm$ SEM (n = 4 - 6) of 3 - 4 independent samples. **[C]** Top panel shows the representative immunoblots of phosphorylated ERK 1/2 (pERK-1/2) and total ERK 1/2 in 3T3-L1 adipocytes incubated in the serum-free media for 30 min, without (Con as control) or in the presence of PD123319, or AngII plus PD123319, or ZD7155, or AngII plus ZD7155. The lower panel shows the statistics (mean $\pm$ SEM, n = 5) of optical density of the pERK-1/2 blots (a.u.) normalized by optical density of the total ERK 1/2 blots. Student's *t*-test: * *p* < 0.05, ** *p* < 0.01 vs. control; # *p* < 0.05 vs. AngII plus PD123319 treatment.

5.7 Production of APJ receptor (apelin receptor) is differentially regulated by AT₁ and AT₂ receptors

APJ receptor (a G protein-coupled receptor also known as AJP or apelin receptor) is expressed in various cell types including adipocytes (Wei et al., 2005). However, how expression of AJP is regulated in adipocytes is completely unknown. Using western blot analysis, we analyzed the effects of AngII, through either AT₁ or AT₂ receptor, on intracellular APJ receptor production in 3T3-L1 adipocytes. Consistent with the molecular weight of APJ receptor (Iwanaga et al., 2006), we detected immunoblot band of ~ 40 kDa from the whole cell lysates of 3T3-L1 adipocytes. As shown in Fig.5.6A, AT₁ receptor stimulation (100 AngII plus 1 μ M PD123319) for 24 hr significantly enhanced APJ receptor production whereas AT₂ stimulation (100 nM AngII plus 1 μ M ZD7155) suppressed it. In the presence of MAPK kinase inhibitor (PD98059), the stimulatory effect of AT₁ receptor was abolished (Fig.5.6B), indicating that MAPK/ERK signaling pathway is involved in AT₁ induced APJ receptor production. But PD98059 did not alter the basal APJ expression or AT₂ activation induced inhibition.

On the other hand, cell-permeable adenylyl cyclase activator (forskolin) (Fig.5.6C) or membrane permeable cGMP (8-bromo-cGMP) (Fig.5.6D) was able to rectify the inhibitory effect of AT₂, but not to the stimulatory effect of AT₁. Without stimulating AngII receptors, forskolin or 8-bromo-cGMP alone potentiated the production of APJ receptors. Interestingly, the results shown in Fig. 5.3, 5.5, and 5.6 suggest that APJ receptor expression and apelin secretion are similarly regulated by the two distinct pathways mediated by AT₁ receptors and AT₂ receptors, respectively.

Figure 5-6 APJ receptor (apelin receptor) expression in 3T3-L1 adipocytes is differentially regulated by AT₁ and AT₂ receptors [A]; and the stimulatory effect of AT₁ activation is MAPK kinase dependent [B] while the inhibitory effect of AT₂ activation is cAMP [C] and cGMP [D] dependent. The doses of all the chemicals are the same as used in Fig.3 and Fig.5. Top panels show the representative immunoblots of APJ under different conditions (24 hr incubation). The lower panels show the statistics (mean ± SEM, n = 5 or 6) of optical density of the APJ blots (a.u.) normalized to optical density of the GAPDH blots from 3-4 independent cell lysate samples. Student's *t*-test: * *p* < 0.05 vs. control; # *p* < 0.05, comparison between the indicated pairs.

5.8 Conclusion and Discussion

Adipocytes play a central role in energy metabolism through the secretion of adipokines which are in turn regulated by other metabolic factors, such as, insulin (Boucher et al., 2005) and catecholamines (Chapter 4) (Than et al., 2011a). Here, we demonstrate that apelin secretion in 3T3-L1 adipocytes is differentially regulated by angiotensin type 1 and type 2 receptors.

Figure 5-7 Illustration of AT₁ and AT₂ signaling pathways on regulation of apelin secretion and expression as well as APJ receptor (apelin receptor) expression in 3T3-L1 adipocytes.

Our results can be fitted into the signaling map illustrated in Fig.5.7. AT_1 receptor is known to couple with G-protein cascade which, in turn, activates phospholipase C- β (PLC- β) (Griendling et al., 1996; Dinh et al., 2001). PLC- β cleaves phosphatidylinositol 3,4-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), and DAG together with IP₃-induced intracellular release of Ca^{2+} subsequently activates protein kinase C (PKC). In adipocytes and other cells, PKC can stimulate MAPK kinase which phosphorylates ERK 1/2 (Ranganathan et al., 2002; Fricke et al., 2004). Activation of ERK 1/2 finally leads to increase of apelin expression and secretion. Such scenario of signal transduction is supported by our observations when AT_1 receptors were selectively activated by blocking AT_2 receptors. Specifically, 1) AngII induced a rise of intracellular Ca^{2+} in the absence of external Ca^{2+} (Fig.5.4C); 2) depletion of internal Ca^{2+} , but not external Ca^{2+} , suppressed the basal and AngII stimulated apelin secretion (Fig.5.4B); 3) activating PKC using the DAG analogue (PMA) increased basal apelin secretion while inhibition of PKC largely abolished AngII induced apelin secretion (Fig.5.4A); 4) AngII stimulated phosphorylation of ERK 1/2 (Fig.5.5C); 5) inhibition of MAPK kinase abolished AngII enhanced apelin secretion and expression (Fig.5.5A and 5B). Considering the fact that in all secretory cells PKC can potently and acutely stimulate secretion from readily releasable pools (Morgan et al., 2005; Xue et al., 2009; Than et al., 2011b), it is likely that the observed acute effect of AT_1 activation on apelin secretion is through MAPK/ERK independent PKC activities. In parallel to the PLC- β pathway as described above, AT_1 activation may also stimulate MAPK kinase through PLC- γ (Schieffer et al., 1996). But this alternative path (if exists in 3T3-L1 adipocytes) appears to be insignificant, because inhibition of PKC totally abolished AngII induced apelin secretion. The importance of PKC and MAPK kinase on apelin secretion was also demonstrated by a previous study, which showed that inhibition of PKC and MAPK kinase blocked the insulin-induced apelin expression in mouse adipocytes whereas PMA enhanced it (Boucher et al., 2005).

AT₂ signaling is distinct to that of AT₁ (Kaschina and Unger, 2003). As shown here, opposite to the stimulating effect of AT₁ signaling, it inhibits apelin secretion. Activation of AT₂ receptor, which is also G-protein coupled, is known to decrease cAMP and cGMP levels by inhibiting adenylyl cyclase (Zhang and Pratt, 1996; Hansen et al., 2000) and guanylate cyclase (Sumners et al., 1991; Takekoshi et al., 2002) respectively. Decrease of cAMP or cGMP level leads to suppression of protein kinase A (PKA) activities or protein kinase G (PKG) activities. As a consequence, apelin expression and secretion are reduced. Such scenario of signal transduction is supported by our observations when AT₂ receptors were selectively activated by blocking AT₁ receptors. Specifically, both adenylyl cyclase activator (forskolin) and membrane permeable cGMP analogue (8-Bromo-cGMP) were able to increase basal apelin secretion and expression, and block the inhibitory effect of AT₂ activation (Fig.5.3). In addition to the cAMP and cGMP pathways, AT₂ activation can stimulate various phosphatases, resulting in reduction of ERK 1/2 phosphorylation (Fig.5.5C) (Dinh et al., 2001). This may also partly account for the inhibitory effect of AT₂ receptors.

The differential regulations by AT₁ and AT₂ receptors underlie the observed biphasic dose-dependent response of apelin secretion to AngII. This phenomenon has also been observed in other cells (Zheng et al., 2003; Utsugisawa et al., 2005). For example, high-dose AngII inhibits fluid transport in rat jejunum through actions of AT₁ receptors, while low-dose AngII stimulates it *via* AT₂ receptors (Jin et al., 1998). Similarly, we show that low-dose AngII inhibits apelin secretion through AT₂ receptors whereas high-dose AngII stimulates it through AT₁ receptors. The low-dose responsiveness (sensitivity) of AT₂ receptor may be ascribed to its higher binding affinity to AngII (Cox and Rosenfeld, 1999; McMullen et al., 2002) and its dominant abundance in 3T3-L1 adipocytes (Jones et al., 1997a; Hung et al., 2010). The concentration of AngII and the expression level of AT₁ or AT₂ receptors change in different physiological and pathological conditions. For instances, AngII concentration is elevated in obesity and hypertension (Aneja et al., 2004; Kotsis et al., 2010); AT₁ receptor expression is up-regulated in obesity (Gorzelnia et al., 2002); and AT₂ receptor is up-regulated during adipo-differentiation of 3T3-L1 as well as human pre-adipose cells (Schling, 2002; Hung et

al., 2010). The co-existence of the antagonizing AT₁ and AT₂ signaling pathways allows the cells to respond differently to different situations. In contrast to 3T3-L1 adipocytes, AT₁ receptors dominate in human adipocytes and other cells in adult tissues (Engeli et al., 1999; de Gasparo et al., 2000). Due to its low expression, the physiological and clinical significance of AT₂ receptor is largely overlooked. However, mounting evidence indicates that expression of AT₂ receptors increases in various pathological conditions and in response to stimulation of several agents including AngII and insulin (Dinh et al., 2001; Lemarie and Schiffrin, 2010).

Figure 5-8 Angiotensin receptor type 1 and 2 (AT₁ and AT₂) differentially regulate the exocytosis of apelin vesicles. **[A]** Confocal image 3T3-L1 adipocytes immunostained with rabbit anti-apelin antibody (Santa Crus Biotech) together with anti-rabbit secondary antibody conjugated with Atto 647 NHS (Sigma-Aldrich). **[B]** TIRFM image of 3T3-L1 adipocytes overexpressed with apelin-tGFP. GFP tagged apelin can be seen as bright dots. **[C]** Sequential images of fusion event of GFP tagged apelin vesicle. The scale bar = 1 µM. **[D]** Activation of AT₁ and AT₂ receptors differentially regulate the exocytosis (fusion events) of apelin vesicles. Cells were 30 min pre-incubated without (Con as a control), or with 100 nM insulin, or 1 µM AngII plus 10 µM PD123319 or 10 nM AngII plus 1 µM ZD7155 before TIRFM experiments. The values represent mean± SEM (5-6 cells from 3-4 separate experiments). Student's *t*-test: ** *p* < 0.01, * *p* < 0.05 vs. control.

Figure 5-9 Compartmentalization of apelin molecules in 3T3-L1 adipocytes revealed by confocal imaging. Upper lane shows the distribution of immunostained apelin (left, Atto 647 conjugated secondary antibody) and overexpressed Glut4 (middle, Glut4-GFP overexpression) molecules, and their co-localization (right, merge of left and middle). Lower lane shows the distribution of immunostained apelin (left, FITC conjugated secondary antibody) and overexpressed Rab5 (middle, REP-Rab5 overexpression), and their co-localization (right). Scale bar = 10 μ m.

Figure 5-10 Apelin release from 3T3-L1 adipocytes was inhibited by the classical secretory pathway inhibitor, Brefeldin A. Top panel shows the representative immunoblots of apelin released to the serum-free media in 2 hr duration, without (Con, as a control) or in presence of 5 μ g/ml or 10 μ g/ml of Brefeldin A. The bottom panel shows the statistics (mean $\pm$ SEM, n = 5) of the optical density of the blots in arbitrary unit (a.u.), normalized to the optical density of GAPDH, from 3-4 independent experiments. Student's *t*-test: * *p* < 0.05 vs. control.

The AngII regulation on apelin secretion cannot be solely attributed to the alteration on protein production which is a slow process. We therefore speculate that apelin molecules may be packed into abundant readily releasable vesicles to support acute secretion and regulation. Several adipokines have been shown to segregate in vesicles (as shown in Chapter 3 and 4) (Xie et al., 2008a; Ye et al., 2010). Using confocal imaging and total internal reflection fluorescence microscopy (TIRFM), we indeed found that apelin molecules are also packed into numerous intracellular vesicles (Fig.5.8). Note that apelin molecules are not co-localized with Glut4, while co-localized with Rab5 containing early endosomes (Fig.5.8.1). In addition, we found that apelin secretion was significantly reduced by Brefeldin A which blocks vesicular traffic between the endoplasmic reticulum and Golgi (Fig.5.9). These suggest that apelin is secreted through the classic vesicular secretory pathway. In contrast to the biochemical assays or electrophysiological assays (Chen and Gillis, 2000; Chen et al., 2001), TIRFM (as shown in Chapter 3 and 4) is able to visualize the individual secretory vesicle in the thin subplasmalemmal region (<200 nm) and resolve the events of vesicular fusion (release) (Zhang et al., 2009b; Soo et al., 2010; Than et al., 2011b). As shown in Fig.5.8C and D, activation of AT₁ receptors significantly increased the frequency of the fusion events of apelin vesicles, whereas activation of AT₂ decreased it.

Autocrine regulations of several adipokines (e.g., leptin and resistin) on adipocytes have been reported (as shown in Chapter 3) (Karastergiou and Mohamed-Ali, 2010; Ye et al., 2010). Several lines of evidence have shown that apelin influences adipocyte functions, such as, lipolysis and glucose uptake (Dray et al., 2008; Yue et al., 2011). And APJ receptors are known to be expressed in adipocytes (Wei et al., 2005; Castan-Laurell et al., 2008). These suggest the autocrine regulations of apelin on adipocytes through APJ receptors. It has been shown that anti-lipolysis effect of apelin on adipocytes is absent in APJ receptor null mice (Yue et al., 2011). Here, we demonstrate that APJ receptor expression and apelin secretion share common AT₁ and AT₂ signaling pathways, implying the intimate collaboration between apelin and APJ receptor. In support of this notion, we also discovered that either application of

apelin or activation of AT₁ receptor prevents β -adrenergic agonist induced lipid droplet fragmentation (data not shown)

Adipose tissue is highly vascularized allowing significant cross-talks between apelin-secreting adipocytes and AngII-secreting vascular endothelial cells (Schling and Loffler, 2002). It has been reported that apelin promotes angiogenesis in adipose tissue (Kunduzova et al., 2008), which in turn may facilitate adipose tissue growth (Schling and Loffler, 2002). Increased vascularization may lead to higher local AngII level. And activation of AT₁ by high level of AngII would stimulate apelin secretion. In addition, it has been shown that AngII can stimulate human visceral adipocyte growth *via* AT₁ receptor (Sarzani et al., 2008). Increased adiposity shall then lead to higher local apelin level. Taken together, a positive feedback loop involving apelin and AngII/AT₁ receptor seems to exist to facilitate adipose tissue growth. This notion may provide mechanistic insights to the common association between obesity and hypertension. The counteracting AT₂ signaling shall provide a route to balance or rectify the positive loop constituted by apelin and AT₁ signaling. Interestingly, it has been reported that AT₁-receptor-blocking anti-hypertension drugs (e.g., valsartan, telmisartan) can reduced adipocyte size, adipose tissue mass and body weight gain in animal models (Mori et al., 2007; Munoz et al., 2009; He et al., 2010) and human (Kakuma, 2009). These may be attributable to the de-shadowing of AT₂ influences in the absence of AT₁ activation. It is conceivable that AT₂ receptor could be a novel therapeutic target to treat obesity and obesity induced hypertension.

In conclusion, this study reveals a new regulatory mechanism on apelin secretion from adipocytes. Based on the present findings, it may be pictured that, determined by the intriguing balance between the counteracting AT₁ and AT₂ signaling, AngII modulates adipocyte functions (e.g., lipolysis and glucose uptake) *via* autocrine regulation by apelin-APJ system and influences metabolic functions of other cells at paracrine and endocrine levels through released apelin.

5.9 Acknowledgement

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CHAPTER 6 APELIN INHIBITS ADIPOGENESIS AND LIPOLYSIS THROUGH DISTINCT MOLECULAR PATHWAYS

6.1 Introduction

Apelin is a peptide hormone identified as the only endogenous ligand for the previously orphaned G protein-coupled APJ receptor (Tatemoto et al., 1998). As apelin and APJ receptor are widely expressed in various tissues and organ systems (e.g., brain, heart, stomach, pancreas, skeletal muscle and blood vessels) (Masri et al., 2005), it is not surprising that apelin is involved in many physiological functions, such as, fluid homeostasis (Galanth et al., 2012), cardiac muscle contraction (Ashley et al., 2005), vascular smooth muscle cell proliferation (Li et al., 2008), and vasodilatation (Japp et al., 2008). More recently, it has been discovered that apelin and APJ receptors are also expressed in adipocytes (Castan-Laurell et al., 2005; Wei et al., 2005).

Adipocyte, which is previously regarded as the passive energy storage site, has now been increasingly recognized for its pivotal roles in metabolic homeostasis via secretion of a variety of signaling molecules called adipokines (Fruhbeck et al., 2001). Recent studies have revealed the emerging roles of apelin in metabolism (Castan-Laurell et al., 2011). Apelin regulates feeding behavior (Valle et al., 2008; Lv et al., 2012), increases insulin sensitivity and glucose uptake (Attane et al., 2010; Yue et al., 2010), and stimulates energy expenditure (Higuchi et al., 2007; Yamamoto et al., 2011). And it is believed to link with metabolic disorders because its plasma level is elevated in type 2 diabetes and obesity (Heinonen et al., 2005; Li et al., 2006; Soriguer et al., 2009).

In Chapter 5, we studied a new regulatory mechanism on apelin secretion and APJ receptor expression in adipocytes. Co-expression of apelin and APJ receptor in adipocytes also implies the autocrine regulations of apelin on adipose tissue. Indeed, it has been shown that body

adiposity and serum level of triglyceride decrease in mice after injection of apelin (Higuchi et al., 2007). Also as recently reported by Yue et al. (Yue et al., 2011), abdominal adiposity and serum level of free fatty acids (FFAs) significantly increase in apelin-null mice while apelin treatment attenuates the isoproterenol-induced FFA release from adipocytes. Likely, apelin is implicated in adipogenesis and lipolysis in adipocytes through yet unknown molecular pathways.

In Chapter 6, we provide evidences that apelin inhibits both adipogenesis and lipolysis, and detailed molecular pathways are proposed. The mouse cell line 3T3-L1 was used because its pre-adipocytic state is a well-established in-vitro culture model for adipocyte differentiation and its differentiated state is a widely-used in-vitro model of mature adipocytes (Green and Kehinde, 1975; Rosen and Spiegelman, 2000).

6.2 Apelin suppresses the progressive expressions of the adipogenic transcriptional factors and prevents the regressive expression of anti-adipogenic factor during 3T3-L1 differentiation

The mouse 3T3-L1 pre-adipocytes, which differentiate into mature adipocytes upon hormonal stimulation, were used as the in-vitro model of adipogenesis (Green and Meuth, 1974; Mandrup and Lane, 1997). It is well-known that adipogenic differentiation is controlled by a set of adipogenic transcriptional factors (Farmer, 2006). Among them, peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer binding protein α (C/EBP α) are known to be critical activators and commonly used as the makers of adipogenesis (Rosen et al., 1999; Rosen et al., 2002). Progressive expression of these activators occurs during adipogenesis. On the other hand, adipogenesis is also synergistically promoted by regressive expression of some anti-adipogenic factors, such as, Wnt10b. Hence, the expression levels of PPAR γ , C/EBP α , and Wnt10b were monitored at different stages of 3T3-L1 differentiation (day 0, 2, 4 and 6 after the initiation of differentiation). As expected, the expressions of PPAR γ and C/EBP α were significantly increased while the expression of Wnt10b greatly declined as the 3T3-L1 cells were differentiating into adipocytes (Fig.6.1).

In adipocytes, preproapelin (77- amino acid long peptide) is expressed by APLN gene and cleaved into shorter active peptides including pyr-apelin13 (pyroglutamated 13 amino acid peptide fragment) (Tatemoto et al., 1998; Kawamata et al., 2001; Than et al., 2012a). Note from Fig.6.1, A and B, that application of pyr-apelin13 to the culture medium considerably suppressed the expression of these transcriptional factors. In contrast, apelin treatment significantly prevented the regression of Wnt10b expression in the early stage of adipocyte differentiation (Fig.6.1C). Given the critical importance of these factors, our experiments suggest the inhibitory regulation of apelin on adipogenesis.

Figure 6-1 During 3T3-L1 adipogenic differentiation, apelin suppresses the expression of adipogenic transcriptional factors (PPAR γ and C/EBP α) and stimulates the expression of anti-adipogenic factor (Wnt10b). Western blot analyses of PPAR γ (~54 kDa), CEBP α (~42 kDa), and Wnt10b (~43 kDa) expression at different stages of differentiation, with (+) or without (-) pyr-apelin13 treatment (1 μ M). The top panels show the representative immunoblots. In [A and B], the lower panels show the statistical data (mean $\pm$ SEM from 5 - 6 independent experiments) normalized to the expression level at day 2 without pyr-apelin13 treatment (as control). In [C], the lower panel shows the statistics (mean $\pm$ SEM from 3 - 4 independent experiments) normalized to the expression level at 30min without pyr-apelin13 treatment (as control). Each sample contains the same amount of proteins (as the internal control). Student's *t*-test: * *p* < 0.05, ** *p* < 0.01 vs. control; # *p* < 0.05, ## *p* < 0.01 between indicated pairs.

6.3 Apelin inhibits the adipogenesis of 3T3-L1 pre-adipocytes

Adipogenesis is a complex process by which fibroblast-like undifferentiated pre-adipocytes transform into morphologically rounded mature adipocytes that accumulate lipid droplets (Gregoire et al., 1998; Rosen and MacDougald, 2006). Lipid droplets accumulation, which is commonly used as the indicator of the degree of adipogenesis, was visually assessed by Oil-red O staining and quantitatively analyzed by measuring intracellular triglyceride. On day 6, most of the 3T3-L1 cells were differentiated into mature adipocytes as evidenced by their morphological change and intracellular lipid droplets stained by Oil-red O (Fig.6.2A). Also as shown in Fig.6.2A, treatment of pyr-apelin13 during differentiation led to a marked decrease of Oil-red O staining (thus lipid droplets accumulation) in a seemingly dose-dependent manner. Consistently, the intracellular triglyceride (TG) content of the differentiated 3T3-L1 cells (day 6) was also significantly and dose-dependently attenuated by the apelin treatment (Fig.6.2B). As shown in Fig. 6.2C and D, the decrease of triglycerides is linked to the decrease of lipogenesis due to apelin-inhibited expression of fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). As FAS and ACC are also adipogenic markers (adipogenesis is accompanied by lipogenesis), these observations further supports our hypothesis that apelin suppresses the 3T3-L1 differentiation into adipocytes.

PPAR γ and C/EBP α are important early markers of adipogenesis while adipocyte protein 2 (aP2) is a late marker. As shown in Fig. 6.2E, apelin also inhibited the expression of aP2. Leptin is an adipocyte-specific hormone, which can also serve as a specific marker of mature adipocytes or adipogenesis (MacDougald et al., 1995; Liu et al., 2011b). Expression of leptin

is not detectable in 3T3-L1 pre-adipocytes. As shown in Fig.6.2F, leptin expression drastically increased over time during the differentiation process, and it was largely inhibited by apelin. Taken together, the inhibitory role of apelin on adipogenesis is established by (1) its prevention on the regressive expression of anti-adipogenic factor (Wnt10b) and progressive expression of adipogenic transcriptional factors (PPAR γ and C/EBP α), and (2) its down-regulations of lipid droplet formation, intracellular triglycerides, and expressions of adipogenic markers (ap2, leptin FAS, and ACC).

As apelin is an adipokine secreted by adipocytes (Boucher et al., 2005) and APJ receptors are expressed in pre-adipocytes and adipocytes (Wei et al., 2005; Rayalam et al., 2008), it is natural to propose that adipogenesis is inhibited by the autocrine regulations mediated by apelin and APJ receptor interaction. Interestingly, it was found that application of exogenous apelin promotes APJ receptor expression during adipogenesis (Fig.6.2H). Therefore, it is conceivable that the autocrine inhibitory regulations by apelin/APJ receptor increase over time, serving as a negative feed-back control to adipogenic process. The fact that secretion of apelin is increased over time during differentiation (Fig.6.2G) further support this negative feedback control.

6.4 Apelin overexpression suppresses the adipogenesis while knocking down of apelin gene expression accelerates it.

The exogenous application of apelin was found to suppress the adipogenesis (Fig.6.1 and 6.2). To corroborate the hypothesis of autocrine inhibition of apelin on adipogenesis, we investigated the effects of apelin overexpression and siRNA knock-down of apelin expression. The overexpressed apelin molecules are tagged with green fluorescence protein (GFP) as the reporter. Similar to the exogenous application of apelin and as anticipated, overexpression of apelin decreased lipid droplet formation, intracellular triglyceride, expression of adipogenic transcriptional factors, and expression of leptin (Fig.6.3). On the other hand, siRNA knock-down of apelin expression produced opposite effects. In control experiments, overexpression

of GFP itself and transfection of control siRNA showed no appreciable effects. Furthermore, exogenous apelin and plasmid transfection did not affect the viability of the cells (Fig.6.3E). Note that apelin release and expression were increased in 3T3-L1 cells overexpressed with apelin while decreased in siRNA knock-down cells (Fig.6.3.1).

Figure 6-2 Apelin inhibits adipogenesis of 3T3-L1 cells. [A] Cells were induced to differentiate into mature adipocytes in the presence of various doses of pyr-apelin13 (0, 0.01, 0.1, 1 μM), and subjected to Oil Red O staining on day 6. [B] Intracellular triglyceride content (mean ± SEM, n = 4 – 5 experiments) in differentiated 3T3-L1 cells (on day 6) treated with different concentrations of pyr-apelin13 (0 μM, as control). [C, D and E] Western blot analyses of FAS (~270 kDa), ACC (~280 kDa), and aP2 (~15 kDa) expression in differentiated 3T3-L1 cells (on day 6) treated with various doses of pyr-apelin13. The top panels show the representative immunoblots while the lower panels show the statistical data (mean ± SEM from 4 - 5 independent experiments) normalized to the expression level without pyr-apelin13 treatment (as control). [F and H] Western blot analyses of leptin (~16 kDa) and APJ receptor (~42 kDa) expression at different stages of differentiation (day 2, 4, 6), without

or in the presence of 1 μ M pyr-apelin13. The top panels show the representative immunoblots, and the lower panels show the statistical data (mean $\pm$ SEM from 5 - 6 experiments) normalized to the expression level (at day 2) without pyr-apelin13 treatment (as control). Each sample contains the same amount of proteins (as the internal control). [G] Enzyme immunoassay (EIA) of extracellularly released apelin (in 6 hr) at different differentiation stages (day 0, 2, 4, 6) (apelin release at day 0 as control). The data is presented as mean $\pm$ SEM (n = 4 - 5). In [B-H], Student's *t*-test: * *p* < 0.05, ** *p* < 0.01 *** *p* < 0.001 vs. control; # *p* < 0.05, ## *p* < 0.01 between indicated pairs.

Figure 6-3 Apelin overexpression suppresses the adipogenesis of 3T3-L1 cells, while knocking down of apelin expression accelerates it. 3T3-L1 cells were induced to differentiate without (control) or with exposed to 1 μ M pyr-apelin13. Similarly, cells either overexpressed with GFP (Control overexpression, ‘3’) or apelin-GFP (Apelin overexpression, ‘4’), or either transfected with control siRNA (Control siRNA, ‘5’) or apelin siRNA (Apelin siRNA, ‘6’), were induced to differentiate into adipocytes. **[A]** Representative Oil-red O staining images (n = 4 – 5 separate experiments), **[B]** triglyceride accumulation level (mean $\pm$ SEM, n = 4 - 5), and **[C]** quantification of Oil-red O staining (OD: optical absorbance at 500 nm; mean $\pm$ SEM, n = 4 - 5), of differentiated adipocytes (on day 6) are shown accordingly. **[D]** Representative immunoblot of expression of adipogenesis-related proteins, PPAR γ , C/EBP α and leptin (on day 6), and their relative expression levels (mean $\pm$ SEM, n = 5 - 6) are shown accordingly. Western blot assays were analyzed using the same amount of protein concentration. **[E]** Effect of 1 μ M pyr-apelin13 or different transfections on the cell growth of 3T3-L1 cells (48 hr incubation) were analyzed using a MTT-based cell growth determination kit (OD at 570 nm, mean $\pm$ SEM, n = 4 - 5). Student’s *t*-test: * *p* < 0.05, ** *p* < 0.01 vs. respective control.

Figure 6.3.1 **[A]** Enzyme immunoassay (EIA) analyses of apelin secretion (in 12 hr) from 3T3-L1 adipocytes (day 6-7 after initiation of differentiation), overexpressed with GFP or apelin-GFP, or transfected with control siRNA or apelin-siRNA. **[B]** Western blot analyses of expressions of apelin-GFP (~40 kDa) or apelin (~16 kDa; a preproapelin dimer) in 3T3-L1 adipocytes (day 6-7), overexpressed with GFP or apelin-GFP, or transfected with control siRNA or apelin-siRNA. In **[A and B]**, the statistics (mean $\pm$ SEM) is obtained from 3 - 4 independent experiments. Student’s *t*-test: * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.01 vs. respective control.

6.5 Apelin-suppressed 3T3-L1 adipogenesis is MAPK kinase/ERK dependent

Previous studies have provided evidence that apelin may stimulate AMP activated protein kinase (AMPK) in adipocytes (Dray et al., 2008; Attane et al., 2010). And studies have also shown that activation of AMPK blocks the expression of PPAR γ and C/EBP α (Habinowski and Witters, 2001; Dagon et al., 2006). Therefore, it is conceivable that apelin suppresses adipogenesis in AMPK dependent manner. To test this possibility, we used dorsomorphin (a potent and selective inhibitor of AMPK) to block the AMPK activity during 3T3-L1 adipocyte differentiation (Zhou et al., 2001). But as shown in Fig.6.4, dorsomorphin (1 μ M) did not promote adipogenesis of 3T3-L1 cells based on both visual inspection on the number of differentiated adipocytes (cells containing lipid droplets and the measurements of PPAR γ and C/EBP α expression levels. And it was also not able to alleviate the inhibitory effects of apelin. Therefore, apelin induced inhibition on adipogenesis is AMPK independent.

Several lines of evidence have shown that increased MAPK kinase/ERK activities suppress adipogenesis (Miyaoaka et al., 2006; Kim et al., 2007; Rhee et al., 2008). And it has been demonstrated that apelin activates ERK phosphorylation through the G-protein coupled APJ receptors in a number of cell types (Masri et al., 2002; Bai et al., 2008). Therefore, we hypothesized that apelin inhibits adipogenesis through stimulating MAPK kinase/ERK pathway. This is indeed supported by the observations that PD98059 (a specific inhibitor of MAPK kinase; 50 μ M), rectified the inhibitory effects of apelin on the number of lipid-droplet containing adipocytes and the expression of adipogenic transcriptional factors (Fig.6.4). Note, however, that PD98059 showed no significant stimulatory effects on adipogenesis in the absence of apelin. Thus, it can be concluded that apelin induced inhibition of adipogenesis is, at least in part, dependent on MAPK kinase/ERK signaling pathway.

Figure 6-4 Apelin inhibition on adipogenesis is MAPK kinase/ERK dependent. 3T3-L1 cells were induced to differentiate into adipocytes without (Control) or in the presence of 1 μ M pyr-apelin13, or 1 μ M dorsomorphin (AMPK inhibitor), or dorsomorphin plus pyr-apelin13, or 50 μ M PD98059 (MAPK kinase inhibitor), or PD98059 plus pyr-apelin13. **[A]** Representative optical images of differently cultured 3T3-L1 cells (day 6). Scale bars = 80 μ m. **[B]** The number of adipocytes (lipid-droplet-containing cells) per field-of-view (40x) differentiated from differently cultured 3T3-L1 cells (day 6). The data is presented as mean $\pm$ SEM (n = 6 – 7 images from 3 – 4 different culture batches). **[C]** Western blot analyses of PPAR γ and C/EBP α expression (day 2). Each sample contains the same amount of total proteins. The statistics (mean $\pm$ SEM) is obtained from 5 - 6 independent experiments. In B and C, Student's *t*-test: * *p* < 0.05, ** *p* < 0.01 vs. control; # *p* < 0.05, ## *p* < 0.01 vs. pyr-apelin13 treatment alone.

6.6 Apelin increases ERK1/2 phosphorylation in cAMP/PKA dependent manner

To further confirm that apelin inhibits adipogenesis by stimulating MAPK kinase, we tested the effects of apelin on ERK1/2 phosphorylation in 3T3-L1 cells. As shown in Fig.6.5A, application of exogenous apelin, as expected, stimulated ERK1/2 phosphorylation in 3T3-L1 pre-adipocyte dose-dependently.

As shown in Fig.6.5B, the stimulation of adipogenesis by the hormone cocktail (IDM) caused acute increase of ERK1/2 phosphorylation (within 15 min). But in accordance with the previous reports (Prusty et al., 2002; Bost et al., 2005), such increase is transient, i.e., the level of ERK1/2 phosphorylation restored quickly towards the basal level in the order of a few hours. The low basal ERK activity implies its insignificant role in adipogenesis and explains why inhibition of basal ERK activity by PD98059 had no obvious effect on adipogenesis (Fig.6.4). Interesting, the stimulated ERK1/2 phosphorylation persisted when apelin was co-applied together with the IDM stimulation. This observation together with those in Fig. 4 implies that apelin inhibits adipogenesis by sustaining the MAPK kinase/ERK activities. As shown in Fig. 6.5C, apelin inhibition appears to act, at least in part, through phosphorylating (thus inactivating) the adipogenic transcriptional factor PPAR α .

In some cell types, cAMP activating protein kinase A (PKA) inhibits MAPK kinase/ERK pathway (Dhillon et al., 2002; Davis et al., 2003). Similarly, we observed that KT5720 (a specific PKA inhibitor; 1 μ M) increases ERK phosphorylation in the 3T3-L1 cells (Fig.6.5C). And it has been reported that apelin decreases cAMP accumulation in 3T3-L1 adipocytes (Yue et al., 2011). Therefore, it is likely that cAMP/PKA acts the upstream of MAPK kinase/ERK. In other words, apelin may inhibit adipogenesis by reducing cAMP/PKA inhibition on ERK signaling. This is supported by the observation that co-application of forskolin (an activator of adenylate cyclase; 10 μ M) abolished apelin stimulated ERK1/2 phosphorylation (Fig.6.5D). Furthermore, our conclusion that apelin inhibition is AMPK independent (Fig.6.4) is corroborated by the observation that co-application of dorsomorphin did not suppress the apelin stimulated ERK1/2 phosphorylation (Fig.6.5D). Taken together, it may be proposed that

apelin inhibits adipogenesis *via* promoting ERK1/2 activities in cAMP/PKA dependent manner which, in turn, inactivates the adipogenic transcriptional factor PPAR γ .

Figure 6-5 Apelin increases the phosphorylation of ERK1/2 through the inhibition of cAMP/PKA pathway. [A, B and D] The top panels show the representative immunoblots of phosphorylated form of ERK1/2 (pERK1/2, ~42 - 44 kDa) and total ERK1/2 (ERK1/2, ~42 - 44 kDa) expressed in 3T3-L1 cells. The lower panels show the statistics of optical density ratio between pERK1/2 and ERK1/2 (mean $\pm$ SEM, $n = 5 - 6$). [C] The top panel shows the representative immunoblots of phosphorylated form of PPAR γ (pPPAR γ , 54 kDa) expressed in 3T3-L1 cells, and the lower panel shows the statistics (mean $\pm$ SEM) obtained from 3 - 4 independent experiments. Each sample contains the same amount of total proteins. [A] Cells were incubated in the serum-free medium for 15 min with exposure to different doses of pyr-apelin13 (10nM, 100nM, 1 μ M; 0 μ M as control). [B and C] Cells were incubated in the differentiation medium (DMEM containing 10 μ g/ml insulin, 1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 10% fetal bovine serum), without (control) or with the addition of 1 μ M pyr-apelin13, for different duration (15 min, 2 hr, 8 hr, 24 hr). [D] Cells were incubated in the differentiation medium for 15 min, without (control) or in the presence of 1 μ M pyr-apelin13, or 10 μ M forskolin, or forskolin plus pyr-apelin13, or 1 μ M KT5720 (30 min pre-incubation), or KT5720 plus pyr-apelin13, or 1 μ M dorsomorphin (30 min pre-incubation), or dorsomorphin plus pyr-apelin13. Student's *t*-test: * $p < 0.05$, ** $p < 0.01$ vs. control (no treatment); # $p < 0.05$ vs. pyr-apelin13 treatment alone.

6.7 Apelin increases the size of lipid droplets and perilipin expression through the AMPK dependent pathway

As seen from Fig. 4A, although exogenous apelin largely decreased the number of differentiated adipocytes, the size of the lipid droplets inside the differentiated adipocytes appeared to be enlarged. To better illustrate this phenomenon, single 3T3-L1 adipocytes with or without apelin treatment during differentiation are shown in Fig.6.6A and the statistics on the size of lipid droplet is presented in Fig. 6.6B. This could be resulted from apelin induced inhibition of basal lipolysis. In the support of this view, the release of free fatty acids (FFA) was indeed found to be reduced by apelin (Fig. 6.6C). This is in line with the previously reported observation that long-term infusion of apelin reduced the serum levels of free fatty acid (FFA) and glycerol in apelin-null mice (Yue et al., 2011).

Perilipin, one of the major lipid droplet-coating proteins, is known to stabilize the lipid droplets and reduce the basal lipolysis in adipocytes (Brasaemle et al., 2000b; Brasaemle, 2007). As revealed by immunostaining (Fig.6.6A), the lipid droplets inside the 3T3-L1 adipocyte was surrounded by perilipin coating, and the coating was obviously thickened by the apelin treatment during differentiation. Thus, it is likely that apelin suppresses basal lipolysis by increasing the expression of perilipin. Western immunoblotting confirmed that application

of exogenous apelin and overexpression of apelin enhanced perilipin expression (Fig.6.6D). Consistently, siRNA knocking-down of apelin reduced perilipin expression. Exogenous application of low doses of apelin (10 and 100 nM), however, was not able to obviously affect perilipin expression or lipid droplet size.

As observed from Fig.6.4, apelin induced enlargement of lipid droplets was eliminated by dorsomorphin, suggesting the importance of AMPK activation. In comparison, inhibition of MAPK kinase by PD98059 did not affect the apelin induced enlargement. Western immunoblotting showed that the apelin enhanced perilipin expression was abolished dorsomorphin, but not affected by PD98059 (Fig.6.6E). Taken together, it may be concluded that apelin inhibits basal lipolysis by increasing perilipin expression through stimulation of AMPK while independent of MAPK kinase/ERK pathway.

Figure 6-6 Apelin enlarges the lipid droplet size, decreases release of free fatty acids (FFA), and increases perilipin expression through AMPK signaling. **[A]** Representative optical image (upper lane) of 3T3-L1 adipocytes (day 7-8) and their immunostained images of perilipin expression (lower lane), without (control) or with exposure to 1 μ M pyr-apelin13. Scale bars = 10 μ m. **[B]** The size of lipid droplets in 3T3-L1 adipocytes (day 7-8). The data is shown as mean $\pm$ SEM (n = 300 – 400 droplets in 40 - 50 adipocytes from 3-4 different cultures). Cells were induced to differentiate without (control) or with 1 μ M Pyr-apelin13, or 1 μ M dorsomorphin, or dorsomorphin plus pyr-apelin13, or 50 μ M PD98059, or PD98059 plus pyr-apelin13. **[C]** FFA release (in 12 hr) from 3T3-L1 adipocytes (day 6), incubated in serum-free medium without (control) or with exposure to 1 μ M pyr-apelin13, or pyr-apelin13 plus 1 μ M dorsomorphin. **[D]** Western blot analyses of perilipin (~57 kDa) expression in adipocytes (day 7-8) without (control) or with exposure to 1 μ M pyr-apelin13, or overexpressed with GFP/apelin-GFP, or transfected with control siRNA/apelin-siRNA. **[E]** Western blot analyses of perilipin expression in adipocytes (day 7-8) differentiated in different conditions as described in **[B]**. In **[D and E]**, the top panels show the representative immunoblots; the lower panels show the statistics (mean $\pm$ SEM) obtained from 5 - 6 independent experiments. Student's t-test: * $p < 0.05$, ** $p < 0.01$ vs. respective control; # $p < 0.05$ vs. pyr-apelin13 treatment alone.

6.8 Apelin's inhibitory effect on adipogenesis and stimulatory effect on lipid droplet size are mediated by APJ receptor

It is likely that apelin acts through its interaction with APJ receptors expressed on adipocytes. This hypothesis is tested in a series of experiments using siRNA knock-down of APLNR gene which expresses the APJ receptor. As expected, APJ receptor expression was markedly reduced in APLNR-siRNA knock-down cells (Fig.6.7, A and B). As shown in Fig. 6.8, siRNA knock-down of apelin or APJ receptor expression in 3T3-L1 cells markedly increased the number of differentiated adipocytes and reduced the size of lipid droplets. Furthermore, application of exogenous apelin was able to reverse the effects of knock-down of apelin expression whereas it was not able to reverse the effects of gene knock-down of APJ receptors. These experiments unambiguously demonstrate the critical involvement of APJ receptors.

Figure 6-7 Western blot analyses of APJ receptor (~40 kDa) expression in 3T3-L1 adipocytes, or in rat adipose-derived stem cells. **[A]** 3T3-L1 cells, without transfected (control) or transfected with apelin-siRNA or APLNR-siRNA, were induced to differentiate for 7-8 days. **[B]** Six days after initiation of differentiation, 3T3-L1 adipocytes (without transfected during differentiation) were treated without (control) or with 1 μ M pyr-apelin13, or overexpressed with apelin-GFP for 48 hr. In **[A and B]**, top panels show the representative immunoblots and the low panels show the statistics (mean $\pm$ SEM) obtained from 3-4 independent experiments. Each sample contains the same amount of total proteins. Student's t-test: ** $p < 0.01$, *** $p < 0.001$ vs. respective control. **[C]** Western blot analyses of APJ receptor expression in (a) 3T3-L1 cells and (b) rat adipose-derived stem cells (ADSC).

Figure 6-8 Apelin's inhibitory effect on adipogenesis and stimulatory effect on lipid droplet size are mediated by APJ receptor. Representative optical images of 3T3-L1 adipocytes on day 6-7. Scale bars = 80 μ m (upper lane), 10 μ m (lower lane). 3T3-L1 cells, without transfected (control) or transfected with apelin-siRNA/APLNR-siRNA, were induced to differentiate without or with exposure to 1 μ M pyr-apelin13.

6.9 Apelin ameliorates β -adrenergic agonist induced fragmentation of lipid droplets, perilipin phosphorylation, and FFA release

Upon stimulation, lipolysis can be greatly accelerated by causing fragmentation of lipid droplet, which increases the surface area of lipid droplets available for lipase binding (Ducharme and Bickel, 2008). β -adrenergic agonists can stimulate fragmentation and lipolysis of lipid droplet by activating cAMP/PKA pathway, which, in turn, leads to phosphorylation of perilipin and hormonal sensitive lipase (HSL) (Marcinkiewicz et al., 2006; Brasaemle et al., 2009). As shown in Fig.6.9, epinephrine (β -adrenergic agonist) indeed caused fragmentation of lipid droplets and re-organization of their perilipin coating in 3T3-L1 adipocytes.

Interestingly, co-application of apelin was able to protect lipid droplets (and perilipin coating) from being fragmented by epinephrine. As shown in Fig. 6.9B, apelin partially curbed the epinephrine stimulated perilipin phosphorylation. This can be explained by the previous observation that cAMP elevation induced by isoproterenol (a β -adrenergic agonist) can be reduced by apelin (Yue et al., 2011). Presumably, protective effect of apelin on lipid droplets shall lead to reduced lipolysis. Indeed, it was found that apelin suppressed the epinephrine induced FFA release (Fig. 6.9C). It has been reported that apelin suppresses isoproterenol-induced lipolysis (as determined by the release of free fatty acids); and this is attributed to reduced phosphorylation of HSL due to decreased PKA activation (Yue et al., 2011). Our experiment here implies that the inhibition of apelin on stimulated lipolysis may be attributed in part to reduced fragmentation of lipid droplets by inhibiting phosphorylation of perilipin.

Figure 6-9 Apelin reduces the β -adrenergic agonist induced fragmentation of lipid droplets, perilipin phosphorylation, and release of free fatty acids (FFA) in 3T3-L1 adipocytes. 3T3-L1 adipocytes (day 7-8) were treated without (control) or with 1 μ M epinephrine, or epinephrine plus 1 μ M pyr-apelin13 for 4 hr. **[A]** Representative optical images (upper lane) of adipocytes and corresponding confocal images of perilipin immunostaining (lower lane). Scale bars = 10 μ m. **[B]** The top two panels show the representative immunoblots of perilipin (~57 kDa) and its phosphorylated form (p-perilipin) expressed in adipocytes without (control) or with the treatment (1 hr) of epinephrine and/or pyr-apelin. The lowest panel shows the statistics of optical density ratio between p-perilipin and perilipin (mean $\pm$ SEM, $n = 3 - 4$). **[C]** FFA release (in 4 hr) from adipocytes without (control) or with the treatment of epinephrine and/or pyr-apelin. The statistics (mean $\pm$ SEM) is obtained from 5 - 6 independent experiments. Student's t-test: *** $p < 0.001$ vs. control; ## $p < 0.01$ vs. epinephrine treatment alone.

6.10 Apelin receptors (APJ) surround the lipid droplets

Using confocal microscopy and immunostaining, we examined the cellular localization of APJ receptors in 3T3-L1 adipocytes. As shown in Fig. 6.10, some APJ receptors are associated with the plasma membrane while the majority of them reside intracellularly on the peripheral of the lipid droplets. The coating of APJ receptors on lipid droplets can be three-dimensionally resolved by sequential scanning along Z-axis (Fig.6.11).

Also as shown in Fig. 6.10, apelin treatment or apelin overexpression increased the expression of APJ receptors (in agreement with the Western blot experiments shown in Fig. 6.7), and thickened their coating on the lipid droplets. In addition, it was observed that APJ

receptor coating dispersed upon epinephrine stimulated fragmentation of lipid droplets and apelin treatment counteracted the effect of epinephrine. Interestingly, when the cells were treated with exogenous apelin for 4h, extensive intracellular co-localization of apelin molecules and APJ receptors was observed (Fig.6.12). It may be pictured that binding of apelin to APJ receptors is followed by internalization of apelin-APJ receptor complexes and their translocation onto lipid droplets. These experiments also imply a functional role of apelin-APJ receptor complex directly on lipid droplets.

Figure 6-10 Confocal fluorescence images of a 3T3-L1 adipocyte with immunostaining of APJ receptors. The sequential images (1-8) were taken along Z-axis (with 0.7 μm step). The optical image of the same cell is also shown. Scale bar = 10 μm .

Figure 6-11 Confocal imaging of APJ receptor immunostaining in 3T3-L1 adipocytes (upper lane) and corresponding optical images of the same cells (lower lane). Scale bars = 10 μm. Cells were induced to differentiate without (control, first column) or with exposure to 1 μM pyr-apelin13 (second column), or overexpressed with apelin-GFP (third column). On day7 - 8, adipocytes (without pyr-apelin13 treatment during differentiation) were stimulated for 4 hr with 1 μM epinephrine (forth column) or epinephrine plus 1 μM pyr-apelin13 (fifth column).

Figure 6-12 Co-localization study of apelin and APJ receptor in 3T3-L1 adipocytes. Representative confocal fluorescence images of 3T3-L1 adipocytes (day 7-8) with immunostaining of apelin (green) and APJ receptors (red), [A] without treatment, or [B] with 1 μM pyr-apelin13 treatment for 4 hr (lower lane). Scale bars = 10 μm. Images in the third column are the merged images from column 1 and 2.

6.11 Apelin’s effects on adipogenesis and lipid droplet size in rat adipose-derived stem cells

Adipose-derived stem cells (ADSC), which can differentiate into adipocytes upon hormonal stimulation, have been used as the *in-vitro* cell model for adipogenesis. We found that APJ receptors are also expressed in ADSC, suggesting the existence of apelin/APJ signaling (Fig. 6.7C). As shown in Fig. 6.13, similar to its effects on 3T3-L1 cells, apelin also increases the size of lipid droplets in adipocytes differentiated from ADSCs and inhibits adipogenesis of ADSC as evidenced by reduced number of adipocytes and reduced expressions of leptin and aP2 (late makers of adipogenesis). Hence, the observed apelin effects are not specific only to 3T3-L1 cells.

Figure 6-13 Apelin's effects on adipogenesis and lipid droplet size in rat adipose-derived stem cells (ADSC). ADSCs were differentiated into adipocytes for 10-14 days without (control) or with exposure to 1 μ M pyr-apelin13 during differentiation. **[A]** Representative optical images of differentiated ADSCs. Scale bars = 80 μ m. **[B]** The size of lipid droplets in ADSC-derived adipocytes. The data is shown as mean $\pm$ SEM (n = 200 – 300 droplets in 40 - 50 adipocytes from 3 - 4 different cultures). **[C]** The number of ADSC-derived adipocytes (lipid-droplet-containing cells) per field-of-view (40x). The data is presented as mean $\pm$ SEM (n = 6 – 7 images from 3 – 4 different culture batches). **[D and E]** Western blot analyses of leptin (~ 16 kDa) and aP2 (~15 kDa) expressions in differentiated ADSCs. The top panels show the representative immunoblots and the lower panels show the statistics (mean $\pm$ SEM, n = 5 - 6). Student's t-test: * $p < 0.05$, ** $p < 0.01$ vs. control.

6.12 Conclusion and Discussion

Adipocytes play central roles in regulating metabolic homeostasis via secretion of a variety of adipokines (Fruhbeck et al., 2001). These messengers regulate metabolism in peripheral tissues and influence the secretion and functions of other metabolic factors (as shown in Chapter 4) (Ouchi et al., 2011; Than et al., 2011a). Intriguingly, there are emerging evidences that adipokines exert autocrine regulation on adipocyte functions including adipogenesis, lipolysis, and secretion of other adipokines (Karastergiou and Mohamed-Ali, 2010). For examples, leptin suppresses adipogenesis (Rhee et al., 2008) while adiponectin promotes it (Fu et al., 2005); leptin induces lipolysis (Fruhbeck et al., 1997) while adiponectin reduces it (Qiao et al., 2011); and leptin and resistin suppress each other's secretion (Chapter 3) (Ye et al., 2010). In the present study, we revealed the autocrine regulations of apelin on adipogenesis of 3T3-L1 pre-adipocytes and lipolysis of differentiated 3T3-L1 adipocytes. Our findings can be fitted into the signaling map illustrated in Fig. 6.14.

Figure 6-14 Illustration of signaling pathways involved in inhibitory effect of apelin on adipogenesis and lipolysis in 3T3-L1 cell.

APJ receptors are expressed in adipocytes (Castan-Laurell et al., 2005; Wei et al., 2005). They are believed to couple to both the heterotrimeric Gi (Masri et al., 2002; Moon et al., 2007) and Gq proteins (Szokodi et al., 2002). Upon binding of apelin molecule with APJ, the activated Gi protein inhibits adenylyl cyclase which, in turn, decreases the intracellular cAMP and the activities of protein kinase A (PKA) (Moon et al., 2007; Bai et al., 2008). Suppressed PKA activities relieve their inhibition on the activation of MAPK kinase/ERK signaling (Dumaz and Marais, 2005). Subsequently, enhanced ERK1/2 phosphorylation inhibits the expression of adipogenic transcriptional factors. In addition, stimulated Wnt signaling (Wnt10b) by apelin may also account for the inhibition of the expression of PPAR γ and C/EBP α . As the result of this apelin initiated signaling, adipogenesis is inhibited. Such scenario of signal transduction is supported by our observations (Fig. 6.1 - 6.8) that 1) external application of apelin decreased the progressive expression of critical adipogenic transcriptional factors (PPAR γ and C/EBP α), prevented the regressive expression of anti-adipogenic factor (Wnt10b), decreased the expression of adipocyte-specific markers (aP2, leptin, FAS, ACC) and the number of lipid-droplet-containing mature adipocytes; 2) overexpression of apelin exerted the similar effects as exogenous apelin whereas siRNA knock-down of apelin expression produced the opposite, implying the autocrine actions of apelin after being secreted; 3) siRNA knock-down of APJ receptor expression increased the adipogenesis and this effect could not be counteracted by exogenous apelin treatment; 4) apelin inhibition on the expression of adipogenic factors and adipogenesis was blocked by inhibiting MAPK kinase; 5) external application of apelin enhanced ERK1/2 and PPAR γ phosphorylation; 6) a PKA stimulator (forskolin) suppressed apelin induced ERK phosphorylation while a PKA inhibitor (KT5720) increased ERK phosphorylation. The proposed signaling transduction is also supported by the previous findings that apelin is able to stimulate ERK phosphorylation in other cell types (Masri et al., 2002; Bai et al., 2008). And several studies have shown that sustained activation of ERK1/2 during differentiation restrains adipogenesis (Bost et al., 2005), and drugs or hormones that suppress adipogenesis are also potent activator of ERK1/2 (Miyaoka et al., 2006; Kim et al., 2007; Rhee et al., 2008).

Here, for the first time, we illustrate the inhibitory regulation of apelin on adipogenesis. And it provides a possible explanation to the previous finding in mice that apelin is able to reduce the mass of white adipose tissue (Higuchi et al., 2007). It is likely that apelin serves as a negative feed-back control at autocrine level to restrain adipogenesis (thus increase of body adiposity), because progress of adipogenesis or increase of adipose tissue leads to increase of apelin secretion. And such feedback control is further strengthened by the increasing expression of APJ receptors during the progress of adipogenesis (Fig. 6.2E). Plasma level of apelin is elevated in obesity (Heinonen et al., 2005; Soriguer et al., 2009). Conceivably, obesity may be resulted if the inhibitory regulation of apelin on adipogenesis is disrupted. This idea is consistent with a previous finding that the body fat mass notably increases in APKO mice (mice with generalized deficiency of apelin signaling) (Yue et al., 2011). Our study suggests the possibility to combat obesity by increasing apelin level or the potency of apelin signaling.

In this Chapter 6, we also presented the evidences that apelin is able to inhibit lipolysis by stabilizing the lipid droplets. We observed that 1) exogenous apelin stimulated the expression of lipid-droplet-coating protein (perilipin), enlarged the size of lipid droplets, and reduced the release of free fatty acids (FFA); 2) these apelin effects were blocked by inhibiting AMPK with dorsomorphin; 3) overexpression of apelin acted similarly as external application of apelin while siRNA knock-down of apelin expression acted oppositely; 4) inhibiting AMPK reduced the size of lipid droplets; 5) siRNA knock-down of APJ receptor expression markedly reduced the lipid droplet size and this effect could not be abolished by exogenous apelin treatment. Based on these results, the following transduction may be proposed. Mediated by Gq protein, apelin/APJ interaction activates AMPK; activation of AMPK stimulates the expression of perilipin; the thickened perilipin coating stabilizes the lipid droplets from fragmentation and remodeling, and protects them from being attacked by lipases, and reduces FFA release.

The above picture is also supported by the previous studies. It has been shown that lipid accumulation is markedly reduced in 3T3-L1 adipocytes treated with dorsomorphin (Suenaga

et al., 2010) and, in the mice lacking the predominant α subunit of AMPK, basal lipolysis is considerably increased and the size of adipocytes is reduced (Daval et al., 2005). Perilipin is known to regulate the activity of adipose triglyceride lipase (ATGL), the rate-limiting enzyme for triglyceride hydrolysis (Zimmermann et al., 2004; Wang et al., 2011). And in the perilipin null mice, basal lipolysis is elevated and the size of adipocytes is smaller (Martinez-Botas et al., 2000; Tansey et al., 2001). Under basal condition, perilipin binds the ATGL co-activator, CGI-58, and thereby suppress the interaction of ATGL and CGI-58, and hence inhibit the lipolysis (Granneman et al., 2009). Mutations in perilipin have been recently shown to increase lipolysis by failing to sequester the ATGL and CGI-58 (Gandotra et al., 2011). A number of studies have demonstrated that increase (decrease) of perilipin via overexpression (gene knock-down) or hormonal/chemical stimulation (inhibition) results in reduced (increased) lipolysis and increased (reduced) lipid droplet size (Souza et al., 1998; Tansey et al., 2004; Sawada et al., 2010; Liu et al., 2011a).

β -adrenergic agonists are major lipolytic hormones in the body (Arner, 1999). They activate PKA which, in turn, phosphorylates the hormone-sensitive lipases (HSLs) to facilitate their association with lipid droplets (Krintel et al., 2008; Wang et al., 2009). PKA also phosphorylates perilipin molecules to cause their dissociation from lipid droplets, leading to fragmentation and lipid droplets and thus increased surface area for HSL association (Marcinkiewicz et al., 2006; Granneman et al., 2007). In a previous study, apelin inhibition on hormone-stimulated lipolysis was attributed to the reduction of HSL phosphorylation (Yue et al., 2011). Our experiments here suggest that this can also be attributed to prevention of hormone-stimulated fragmentation of lipid droplets (Fig. 6.9). Apelin inhibition on lipolysis may also be assisted by the direct interaction of APJ receptors with lipid droplets. We observed that APJ receptors, just like other lipid-droplet coating proteins (perilipin, adipophilin) (Blanchette-Mackie et al., 1995; Brasaemle, 2007), densely surround lipid droplets and reorganize upon hormone stimulated droplet fragmentation (Fig. 6.10 and 6.11). And co-localization of apelin and APJ receptors was observed on the peripheral of lipid droplets after external application of apelin, suggesting the internalization of apelin/APJ

receptor complex onto lipid droplets and their direct functions on fragmentation and/or lipolysis. The specific actions on APJ receptors on lipid droplets require further investigations. Taken together, we provide evidences to show that apelin/APJ receptor may regulate lipolysis through three distinct routes.

It has been demonstrated that apelin reduces plasma level of free fatty acids (FFA) and release of FFA from adipocytes (Xu et al., 2011; Yue et al., 2011). Our study provides molecular mechanisms underlying this phenomenon. Specifically, this can be attributed to apelin inhibition on both adipogenesis and lipolysis. It is well known that high FFA level can lead to insulin resistance in peripheral tissues (Boden, 1999, 2001). The present study implies that secretion of apelin and autocrine signaling of apelin in adipocytes may serve as novel therapeutic targets for metabolic disorders, particularly, obesity induced type 2 diabetes.

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CHAPTER 7 GENERAL CONCLUSION AND PERSPECTIVES

7.1 Adipokine secretion is highly regulated, and adipokines function on energy metabolism at autocrine, paracrine and endocrine levels

The identification of adipokines synthesized and secreted from adipocytes (fat cells) has led to the modern view of adipose tissue as a largest endocrine organ. Growing evidences have shown that these adipokines play an important role in regulation of metabolic homeostasis by acting not only on central nervous system, but also on peripheral organs such as adrenal glands, liver, stomach and adipose tissue itself. The secretion of adipokines from adipocytes is highly regulated by other adipokines at autocrine level or by metabolic factors released from other cells at paracrine or endocrine levels.

Using the integrative platform based on total internal reflection fluorescence microscopy, confocal microscopy, carbon fiber amperometry, $[Ca^{2+}]_i$ imaging photometry, molecular biology, immunoblotting and immunoassays, we have studied the vesicular storage and distribution, vesicular trafficking and exocytosis of adipokine secretion, their cross-talks among themselves and with other metabolic factors, and their regulations on adipocyte functions. Specifically, we have demonstrated 1) the similarities, differences, and cross-talks between two important adipokines: leptin and resistin; 2) cross-talks between adipokines (leptin and resistin) and catecholamines; 3) differential regulations of apelin secretion and APJ receptor expression by angiotensin type 1 and type 2 receptors; 4) inhibitory autocrine regulation of apelin on adipocyte differentiation and lipolysis.

In [Chapter 3](#), we provided evidences that leptin and resistin are compartmentalized into different vesicles which are distinct to early endosomes and Glut-4 containing vesicles. We

have shown that both adipokines are released through regulated secretion, since the vesicle trafficking and secretion of leptin and resistin are differentially regulated by insulin/glycolytic substrates and protein kinase A. It indicates that, because of their large number of vesicular storages, both leptin and resistin could utilize regulated secretory pathways (vesicular exocytosis) upon influence of various hormones and substances. Like other neuroendocrine cells, regulated secretion of adipokines in adipocytes is critical, since it can response to acute metabolic conditions. For example, in response to hyperinsulinemic stage after a meal (our experiment condition of insulin/glycolytic substrates mimics this hyperinsulinemic stage), leptin secretion is stimulated that could potentiate the signals of nutritional status to the brain which in turn regulate back on food intake, insulin sensitivity, and energy expenditure. At the same time, reduced secretion of resistin in response to hyperinsulinemia also could enhance the insulin sensitivity in peripheral tissue. Different compartmentalization of leptin and resistin could realize the different secretory pathways in response to the same metabolic condition. It is also not surprising that the two adipokines are inhibited each other, considering that they counteract in metabolic regulations such as insulin sensitivity. Hence, this study, which highlights the several key aspects of secretory pathways adipokines and the factors controlling the secretion of adipokines, could potentiate our understanding of how the adipokines are involved in body energy metabolism.

In Chapter 4, the effect of catecholamine or acetylcholine on adipokine secretion was elucidated. The links between catecholamines, acetylcholine and adipokines may be functionally important. Here, we showed the differential effects of catecholamines and acetylcholine on leptin and resistin secretion. In other words, in contrast to adrenergic stimulation (mainly via norepinephrine), cholinergic stimulation (via Acetylcholine) increased leptin and resistin secretion. Catecholamine is the major neurotransmitter for sympathetic nervous system (which is stimulated for fight-or-flight stress response) while Ach is mainly responsible for parasympathetic nervous system (including vegus nerve; and vagal tone is increased especially during feeding). Interestingly, it was known that white adipose tissue is innervated by both the sympathetic and parasympathetic (Bartness and Bamshad. 1998, Kreier

and Buijs. 2007). Hence, taken together with our results, it might suggest that vagal activity during feeding could produce a stop signal for feeding through the increased adipocyte secretion of leptin (a circulating satiety factor). Disruption of this link could result in overfeeding and obesity. In contrast, stress response could eventually results in increased energy uptake through reduced adipocyte secretion of leptin, and increased insulin-induced glucose uptake of peripheral tissues through reduced ressitin secretion. On the other hand, we also showed that both leptin and resistin could stimulate the secretion of catecholamines (powerful vasoconstrictors responsible for high blood pressure). Since it was known that serum leptin and resistin levels are increased in obese individuals (Kennedy et al. 1997), our results suggest that, obesity (through increase adipokine secretion) could directly contribute to increase blood pressure (or hypertension). Hence, this study not only highlights the importance of adipo-adrenal axis but also the intricate interactions between the various hormones and adipokines.

In Chapter 5, differential regulations of apelin secretion and APJ receptor expression by angiotensin type 1 and type 2 receptors (AT₁ and AT₂ receptor) were investigated. Adipose tissue is highly vascularized allowing significant cross-talks between apelin-secreting adipocytes and AngII-secreting vascular endothelial cells (Schling and Loffler, 2002). In obesity, both apelin and Ang II level is increased in adipose tissue and in circulation (in human adipocytes, majority of AngII receptor is AT₁ type). It has been reported that apelin promotes angiogenesis in adipose tissue through APJ receptor (Kunduzova et al. 2008); and AngII is also known as a potent angiogenic substance through AT₁ receptor. Increased vascularization (angiogenesis) may lead to higher local AngII level which in turn stimulates the apelin secretion through AT₁ receptor. In addition, as shown in Chapter 6, apelin inhibits pre-adipocyte differentiation into mature adipocytes (adipogenesis); and AngII is also known as the anti-adipogenic factor through AT₁ receptor. Hence, a feedback loop involving apelin/APJ receptor and AngII/AT₁ receptor seems to exist in adipose tissue where they both enhance the angiogenic process, while suppress the adipogenic process. The counteracting AT₂ signaling shall provide a route to balance or rectify the effects constituted by apelin and AT₁ signaling.

Hence, our study highlights a new regulatory mechanism on apelin secretion from adipocytes. This study suggests that, by intriguing balance between the counteracting AT₁ and AT₂ receptor signalings, AngII modulates body metabolic functions, such as angiogenesis, adipogenesis, etc. In other words, the signaling pathways mediated by AT₁ and AT₂ receptors may serve as the potential therapeutic targets for the treatment of metabolic disorders, such as obesity and hypertension.

In Chapter 6, inhibitory role of apelin on adipocyte differentiation (adipogenesis) and lipolysis was investigated. It is widely accepted that obesity, which is closely related to insulin resistance in peripheral tissues, increases the risk of diabetes mellitus. It was shown that obesity-associated insulin resistance is likely due to increase plasma level of free fatty acids (FFA) produced from adipocytes (Boden, 1999, 2001). Apelin was known to reduce plasma level of free fatty acids (FFA) and release of FFA from adipocytes (Yue et al., 2011). Here, our study provides molecular mechanisms underlying this phenomenon. Specifically, this lowering of plasma FFA level can be attributed to apelin inhibition on both adipogenesis and lipolysis. Hence, this study implies that secretion of apelin and autocrine signaling of apelin in adipocytes may serve as novel therapeutic targets for metabolic disorders, particularly, obesity induced type 2 diabetes.

In conclusion, study of the adipokine secretion and their functions could definitely enhance our understanding of adipobiology and complex metabolic functions; and such understanding may lead to discovery of potential therapeutic targets or drugs for metabolic diseases, such as obesity and diabetes mellitus.

7.2 Future perspectives

The work presented here only exposes a few fascinating facts related to the regulation of adipokine secretion and their functions which may provide insights to the complex process of metabolism and hormonal interactions. Our studies focused on regulation of leptin, resistin and apelin secretion under the influence of insulin, catecholamines and angiotensin II. Cross-talk between leptin, resistin, and catecholamine secretion was uncovered; and the effect apelin on adipogenesis and adipocyte lipolysis was established in this work. Nonetheless, adipocytes express and secrete a ray of adipokines, and so far, more than 50 adipokines are known, and their number is still growing. Further investigations on the distinct regulatory roles on the secretion and function of adipokines, especially for the newly found ones, chemerin, omentin, vaspin, visfatin, etc., will remain as the major challenges in the future. It is thus of interest to us to investigate how these adipokines are released and regulated, and how these adipokines cross-talk with other metabolic-relevant hormones or metabolites. Obviously, tremendous effects are still needed to explore the roles of these newly found adipokines on metabolic functions.

In addition, adipocytes also express a variety of receptors which could allow binding of respective hormones and substances. Dependence on distinct signaling pathways underlying the different receptor-ligand interactions, various aspects of adipocyte functions, including lipid and glucose metabolism, and the adipokine secretion, will be directed in order to meet the body metabolic homeostasis. For example, AngII, a peptide hormone well-known for its regulation on hemodynamic and metabolic effects, acts through angiotensin type 1 receptor (AT₁) and type 2 receptor (AT₂) (Dinh et al., 2001), both are expressed in human and animal adipocytes (Crandall et al., 1994; Mallow et al., 2000; Weiland and Verspohl, 2008). It was known that angiotensin receptor blockers (AT₁ receptor blocker) reduced the risk for diabetes mellitus (Ribeiro-Oliveira et al., 2008). Recent evidences have shown that AT₁ receptor blocker, (e.g., telmisartan) decrease body weight, triglyceride level, and fat mass in human and rats (Kakuma, 2009; Munoz et al., 2009; He et al., 2010). These observations emphasize the

involvement of AT₁ and AT₂ receptor in metabolic homeostasis by the action of AngII via autocrine, paracrine and endocrine regulation. In Chapter 5, we have shown that AngII differentially regulate on apelin/APJ system in adipocyte through distinct AT₁ and AT₂ signaling pathways. Then, how does AT₁ or AT₂ receptor regulate on secretion of other adipokines? Do these receptors differentially regulate on other adipocyte functions? These questions need to be addressed in the future. Our future plan includes, but is not limited to, the following investigations.

To investigate the differential regulations on adipocyte lipolysis by AT₁ and AT₂ pathways

Previous studies of AngII regulation on lipolysis are controversial. AngII infusion in healthy males had no effect on adipocyte lipolysis activity (Townsend, 2001). No change of lipolysis was observed when AngII was applied to adipose tissue (Boschmann et al., 2002; Boschmann et al., 2003). However, systemic AngII infusion in rats was shown to enhance lipolysis in subcutaneous and visceral fat, while AT₁ receptor blocker (losartan) prevented the effect of AngII on lipolysis (Cabassi et al., 2005). Irbesartan, AT₁ receptor blocker, infusion also reduced the isoproterenol-induced lipolysis in obese man, suggesting the enhancing effect of AngII on lipolysis (Boschmann et al., 2006). In contrast, Goossens et al showed that lipolysis was inhibited when AngII was applied to adipose tissue in normal and obese subjects (Goossens et al., 2004). It was shown that AngII suppressed adipocyte lipolysis was via AT₁ receptor (Goossens et al., 2007).

Given the conflicting effects of AngII on lipolysis, emphasis only on AngII and AT₁ receptor will be oversimplified. Evidences suggest that activation of AT₁ and AT₂ triggers distinct signaling pathways with antagonizing effects (Chung et al., 1996; Jöhren et al., 2004). Therefore, we hypothesize that AngII regulate lipid metabolism through distinct AT₁ and AT₂ signaling pathways in adipocytes, the dominance of which depends on the AngII level and the expression ratio of AT₁ and AT₂ receptors. And hence, we plan to

1) Investigate the differential regulations on adipocyte lipolysis by AT₁ and AT₂ pathways,

2) Investigate the regulations of AT₁ and AT₂ receptor on expression of different lipases (e.g., hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and lipid droplet binding proteins (e.g., perilipin).

To investigate the differential regulations on adipogenesis by AT₁ and AT₂ pathways

Although effects of AngII on adipogenesis and lipogenesis have been studied previously, distinct molecular pathways underlying the AT₁ and AT₂ receptor still need to be elucidated. It was first reported that AngII increased triglyceride content and the activities of two key lipogenic enzymes (fatty acid synthase (FAS) and glycerol-3-phosphate dehydrogenase (GPDH)) in 3T3-L1 and human adipocytes through AT₂ receptor (Jones et al., 1997a). Studies in rodents also suggest the adipogenic effects of Ang II by AT₂ receptor-induced prostacyclin production in mature adipocytes (Darimont et al., 1994; Saint-Marc et al., 2001).

On the other hand, AngII was shown to diminish the insulin-induced lipid accumulation in human pre-adipocytes committed to adipogenesis (Schling and Loffler, 2001). Anti-adipogenic effect of AT₁ receptor in human pre-adipocytes is also reported (Brucher et al., 2007; Fuentes et al., 2010). Similarly, it was shown that AT₁ receptor signaling is involved in mature adipocyte induced inhibition of human pre-adipocyte differentiation (Janke et al., 2002a). In addition, AT₁ receptor blockers induce human in-vitro adipogenesis (Janke et al., 2006), while AT₂ receptor deficiency in arteriosclerotic mice suppresses adipocyte differentiation and decreases adipocyte number (Iwai et al., 2009). Based on these previous observations, we also hypothesize that AngII differentially regulate on adipogenesis and lipogenesis through distinct AT₁ and AT₂ signaling pathways in adipocytes. Therefore, we will further

1) Investigate the differential regulations on adipogenesis by AT₁ and AT₂ pathways

2) Investigate the regulations of AT₁ and AT₂ receptor on expression of adipogenic transcriptional factors (e.g., C/EBP α , PPAR γ) and lipogenic enzymes (e.g., FAS, acyl CoA carboxylase (ACC)).

To investigate the regulations of angiotensin peptides (Ang IV, Ang(1-7)) on lipid metabolism and adipokine secretion

Adipocytes express not only AT1 and AT2 receptors for AngII (Crandall et al., 1994; Mallow et al., 2000; Weiland and Verspohl, 2008), but also express receptors for other angiotensin peptides, for example, AT4 receptor (receptor for AngIV; insulin regulated aminopeptidase(IRAP) is associated with AT4 receptor) (Weiland and Verspohl, 2008), and G-protein coupled Mas receptor (receptor for Ang(1-7) peptide) (Gupte et al., 2008).

In addition, key components of renin-angiotensin system (RAS) are also expressed in adipocytes, for example, angiotensinogen (AGT) (Jones et al., 1997b), angiotensin-converting enzyme (ACE)(Wang et al., 2006) which convert AGT to AngI and then to AngII, and ACE2 (Gupte et al., 2008; Tseng et al., 2010) which convert AngII to Ang(1-7). Formation of AngII, AngIV, Ang(1-7) has been observed in the vicinity of adipocytes (Weiland and Verspohl, 2009). These suggest that local concentrations of various angiotensin peptides may be extremely high. Recently, it was shown that increase in plasma Ang(1-7) level is associated with decrease triglycerides and cholesterol levels, and abdominal fat mass (Santos et al., 2010). Based on these observations, we postulate that, apart from AngII, other angiotensin peptides might also directly regulate on adipocyte functions through yet unknown signaling pathways. Therefore, we aim to

- 1) Investigate the regulation of adipocyte lipolysis by Ang IV and Ang(1-7).
- 2) Investigate the regulation of adipogenesis and lipogenesis by Ang IV and Ang(1-7).
- 3) Investigate the regulation of adipokine secretion by Ang IV and Ang(1-7).

To investigate the regulations of secretion of newly found adipokines and their functions

During the past decade, numerous studies have revealed that adipose tissue secretes a ray of hormones and substances, called adipokines, which have different functions on body metabolism, and hence explained the association between fat mass, obesity and metabolic syndromes.

Vaspin (visceral adipose tissue-derived serine protease inhibitor), a newly found adipokine, has attracted much attention because of its insulin-sensitizing effect in obesity (Bluher, 2012). Vaspin infusion in obese mice improves glucose tolerance and insulin sensitivity (Hida et al., 2005). Increase vaspin serum level is associated with obesity and impaired insulin sensitivity (Youn et al., 2008; Auguet et al., 2011). Vaspin gene expression is much greater in adipocytes than stromal vascular cells of the adipose tissue (Lee et al., 2011).

Omentin is another recently found novel adipokine which is highly localized the omental tissue (visceral adipose tissue). Omentin mRNA is predominantly expressed in stromal vascular cells of the visceral adipose tissues rather than in the fat cells. Plasma omentin level and gene expression are reduced in obesity and diabetes mellitus (de Souza Batista et al., 2007; Tan et al., 2008b). Omentin expression and secretion from human adipose tissue is down-regulated by glucose and insulin (Tan et al., 2008a). Yang et al showed that omentin increases the insulin-stimulated glucose uptake in human adipocytes (Yang et al., 2006).

Apart from these observations, roles of vaspin and omentin on glucose and lipid metabolism are virtually almost unknown. In addition, the mechanism underlying and the regulation on the secretion of these adipokines still need to be elucidated. Therefore, we also intend to

- 1) Investigate the regulation of secretion of vaspin from adipocytes.
- 2) Investigate the possible role of vaspin and omentin on lipid metabolism in adipocytes.

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