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<td>Ye, Feng; Than, Aung; Zhao, Yanying; Goh, Kian Hong; Chen, Peng</td>
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Vesicular storage, vesicle trafficking, and secretion of leptin and resistin: the similarities, differences and interplays

Feng Ye, Aung Than, Yanying Zhao, Kian Hong Goh, Peng Chen*

Running title: The secretion of leptin and resistin

Division of Bioengineering
Nanyang Technological University
70 Nanyang Drive
Singapore 637457

* Corresponding author: chenpeng@ntu.edu.sg

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ABSTRACT

Adipose tissue is a highly active endocrine organ secreting a variety of signaling molecules called adipokines. Leptin and resistin are two adipokines critically involved in metabolic homeostasis. Nevertheless, the secretory pathways of these adipokines and their interplays are poorly elucidated. In this work, we have comparatively studied several key aspects of leptin and resistin secretion from 3T3-L1 adipocytes. It was found that leptin and resistin molecules are compartmentalized into different secretory vesicles. The trafficking of leptin and resistin vesicles, and the secretion of leptin and resistin, are oppositely regulated by insulin/glycolytic substrates and cyclic AMP/protein kinase A. Interestingly, these two adipokines adversely influence each other on secretion and vesicle trafficking. Finally, we demonstrated that both leptin and resistin secretion are Ca^{2+} dependent.
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-alpha, etc.). These adipokines play key roles in regulating energy homeostasis, lipid metabolism, immunity, and other physiological functions (Lago, et al. 2007; Lago, et al. 2009; Rosen and Spiegelman 2006; Trujillo and Scherer 2006). 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 (Dal Rhee, et al. 2008; Kim, et al. 2001; Zhou, et al. 1999). They all encourage angiogenesis and actively regulate immune responses (Anagnostoulis, et al. 2008; Bouloumie, et al. 1998; Lago et al. 2007; Mu, et al. 2006; Reilly, et al. 2005). 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; Steppan, et al. 2001); leptin reduces gluconeogenesis whereas resistin promotes it (Banerjee, et al. 2004; Rossetti, et al. 1997); 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 crosstalks 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.

Methods

Cell culture and differentiation

Murine 3T3-L1 fibroblasts (American Type Culture Collection, MD, USA) were cultured in DMEM medium (Gibco, MD, USA) supplemented with 10% heat-inactivated bovine calf serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO₂/95% air. Differentiation of 3T3-L1 fibroblasts to adipocytes was induced similarly as previously described (Shao and Lazar 1997). Specifically, after cells reached confluence for 2 days (referred as day 0), cells were cultured in the differentiation medium for 2 days, which is DMEM medium supplemented with 10% fetal bovine serum (FBS), 1μM dexamethasone, 10 μg/ml insulin, and 0.5 mM isobutyl-1-methylxanthine. The cells were then maintained in the medium containing 10% fetal bovine serum and 10 μg/ml insulin for 2 days and replenished with the DMEM containing 10% FBS. Differentiation was monitored by the visual appearance of fat droplets in the cells and oil red stain.

Plasmids
The plasmid encoding murine resistin was purchased from OriGene Technologies (Rockville, MD, USA). The plasmid encoding murine leptin was a kind gift from Professor Jan Tavernier (Ghent University, Belgium). The Glut4-GFP plasmid was a kind gift from Professor Jeffrey E. Pessin (Albert Einstein College of Medicine, USA) and the Rab5-GFP plasmid was generously provided by Dr. Sean Liour (Institute of Bioengineering and Nanotechnology, Singapore). The vector pEGFP-N1 was purchased from Clontech (Mountain View, USA). The resistin/leptin-EGFP plasmids were constructed by subcloning the corresponding cDNAs into the vector pEGFP-N1 at the sites of Nhe I and AgeI. Insertion of cDNAs was verified by DNA sequencing.

Differentiated 3T3-L1 cells grown on polylysine coated glass coverslips (on day 7 or 8) were transfected with relevant plasmids (1-2 µg) using FuGENE6 Transfection Reagent (Roche, Indianapolis, USA), 3-4 days before the confocal or TIRFM imaging experiments.

**Confocal Microscopy**

The 3T3-L1 adipocytes were fixed with 4% formaldehyde, permeabilized with 0.1% TritonX-100, blocked with 1.5% bovine serum albumin before immunostaining and imaging. In some experiments, the adipocytes were transfected with plasmids encoding Glut4-GFP or Rab5-GFP 3-4 days before fixation. Leptin molecules were immunostained first with chicken anti-leptin IgY antibody (Abcam, MA, USA) or rabbit anti-leptin IgG antibody (Sigma-Aldrich, MO, USA) and subsequently with the anti-IgY secondary antibody conjugated with FITC (Abcam) or anti-IgG secondary antibody conjugated with Atto 647 NHS (Sigma-Aldrich). Resistin molecules were immunostained first with rabbit anti-resistin IgG antibody (Biovision, CA, USA) and subsequently with the anti-IgG.
secondary antibody conjugated with Atto 647 NHS (Sigma-Aldrich). Confocal images were taken by a LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Germany).

**TIRFM imaging and analysis**

Total internal-reflection fluorescence microscopy (TIRFM) was carried out at 25 ºC using a Zeiss Axiovert 200 inverted microscope system (Carl Zeiss, Germany) equipped with a 100 × 1.45 NA TIRF objective. EGFP was excited by a 488 nm laser and the emission wavelength was collected at 520 nm. The laser beam was incident on the coverslip (refractive index ~1.52) at 68-70° from the normal. The penetration depth of the evanescent field is calculated to be ~100 nm. Using MetaMorph 6.3 program (Molecular Devices, CA, USA), time-lapse TIRFM images were acquired by a CCD camera for 2 min with exposure time of 20 ms and 2 Hz sampling frequency. Vesicles were individually tracked using Image J (http://rsb.info.nih.gov/ij/). The vesicle velocity, dwell time, and arrival rate were analyzed by a program developed in Igor (WaveMetrics, OR, USA). Imaging was taken ~30 min after applying particular chemicals.

**Western blot**

Western blots under different conditions were conducted parallelly (same time and the same batch of cell sub-cultures). And cells were grown confluent in the flasks of same size. Therefore, the cell number for each experiment is similar. On day 9 of differentiation, 3T3-L1 adipocytes were incubated in serum-free medium (Lonza, Switzerland) for 2h followed by medium collection (12 ml) and replenishment with the culture medium. The total amount of secreted proteins in the collected medium was then quantified by a protein assay kit (Bio-Rad, CA, USA) based on the method of Bradford.
This was used to calibrate the sample volume for the following immunoblotting experiments to minimize the influence due to the variation in cell number. In addition, intracellular GAPDH as an indicator of the total cell number was quantified in some experiments. Both measurements of total secreted proteins and intracellular GAPDH on the same batch of cell sub-cultures only exhibited insignificant differences. On day 10 or 11 of differentiation, the cells in 75 cm² flask were washed with PBS for three times and incubated in 12 ml serum-free medium containing 5.5 mM without (control) or with addition of (1) GPI (pyruvate, 1mM; glucose, 25mM as final concentration; and insulin, 100 ng/ml) or (2) GPI and 10 µM forskolin or (3) 10 µM forskolin or (4) 0.1 µM leptin or (5) 0.1 µM resistin. In some experiments, the cells were pre-incubated with intracellular calcium chelator BAPTA-AM (50 µM) in the calcium-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⁺. After 2h, the medium was harvested and concentrated by centrifugal filter devices (Amicon Ultra, Millipore, USA) for western blot analysis. Samples were separated on SDS-PAGE. Proteins were then transferred onto PVDF membranes (Bio-Rad, CA, USA) followed by blocking for 2h at 4°C with 5% non-fat dry milk in TBST buffer (10 mM Tris, 150 mM NaCl, and 0.05% Tween-20, pH 7.5). The PVDF membrane was subsequently incubated with the primary rabbit anti-leptin (Sigma-Aldrich) or goat anti-resistin (Santa Cruz, CA, USA) or anti-EGFP antibodies (Biovision, CA, USA) in TBST buffer at room temperature for 12 h. After washing three times with TBST buffer, the membrane was incubated with a secondary antibody-conjugated horseradish peroxidase (Thermo Scientific, USA) for 6 h at room temperature. The reactions were visualized with CN/DAB detection kit (Thermo
Scientific). PVDF membranes were then scanned using Bio-Rad’s Densitometer and analyzed using Bio-Rad’s QualityOne software. Different rabbit anti-leptin (Santa Cruz), goat anti-resistin (Abcam), and rabbit anti-resistin (Biovision) antibodies were also tested and gave similar results.

[Ca$^{2+}$]$_i$ imaging

3T3-L1 adipocytes were loaded with 5 μM fura2 acetoxy-methyl ester (Fura2-AM, Molecular Probes, OR, USA) for 60 min in culture medium. Then the cells were rinsed and placed in the bath solution for >30 min before the experiment to allow retention of the dye after hydrolysis of the ester. Fluorescent signals in response to GPI, forskolin, calcimycin, or high K$^+$ stimulation were recorded from single cells using a photometry system (TILL Photonics GmbH, Germany). The intracellular calcium concentration ([Ca$^{2+}$]$_i$) was reported as the ratio of Fura2 fluorescence intensity resulting from the two excitation wavelengths at 340 nm and 380 nm, respectively (F340/F380).

Solutions

The bath solution used in confocal, TIRFM and Ca$^{2+}$ imaging experiments contains (in mM, titrated to pH 7.4): 150 NaCl, 5.4 KCl, 2 MgCl$_2$, 2 CaCl$_2$, 5 glucose, and 10 HEPES. Two mM CaCl$_2$ was omitted in the Ca$^{2+}$ free bath solution, which was used to load BAPTA-AM in the immunoblotting experiments. In Ca$^{2+}$ imaging experiments, high K$^+$ solution contains (in mM, titrated to pH 7.4): 40 NaCl, 105 KCl, 1 MgCl$_2$, 6 CaCl$_2$, 5 glucose, 10 HEPES. In some experiments, GPI or 10 μM forskolin or 0.1 μM leptin or 0.1 μM resistin or 1mg/ml calcimycin was added in the bath solution.

Statistical analysis
Statistical data is expressed as mean ± SEM. Because the vesicle dwell time and velocity give skewed distributions, their statistical significances were evaluated by the Mann-Whitney rank sum test (Bai, et al. 2007; Zhou, et al. 2007). All other values were evaluated by Student’s unpaired t-test. $p < 0.05$ was considered to be statistically significant.

**Results**

**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 colocalized (Fig. 1a). Confocal colocalization studies were then performed on 3T3-L1 adipocytes overexpressed with GFP-tagged Rab5 which is a molecular marker of early endosomes. As demonstrated in Fig. 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. 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.
Trafficking of leptin- and resistin vesicles is 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; Xue, et al. 2009a). 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; Xue, et al. 2009b; Zhang et al. 2008).

Subplasmalemmal leptin-vesicles (Fig. 2a) and resistin-vesicles (Fig. 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. 2.

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. 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; Zhang et al. 2008). 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. 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 (19.3 +/- 0.8 s vs. 23.1 +/- 0.9 s, p < 0.05) (Fig. 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. 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 (25.1 +/- 0.8 s vs. 29.3 +/- 0.7 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) by increasing the level of cyclic AMP (cAMP), inhibits insulin stimulated leptin production 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. 3a). In addition, forskolin completely blocked the facilitating effects of GPI, suggesting that cAMP/PKA may act downstream of GPI (Fig. 3a). Conversely, forskolin significantly enhanced the lateral and vertical trafficking of resistin vesicles, and reduced the vesicle dwell time (Fig. 3b). And forskolin counteracted GPI inhibition on vesicle velocity and elongation on dwell time (Fig. 3b). Taken together, our experiments showed that GPI and forskolin differentially regulate the trafficking (thus, likely exocytosis) of leptin- and resistin- vesicles.

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 (27.1 +/- 0.6 s, p < 0.01 vs. control) (Fig. 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 vs. control) and the rate of vesicle delivery (13 +/- 1.6 vesicles arrived in 2 min, p < 0.001 vs. control), and increased the vesicle dwell time (32.8 +/- 0.8 s, p < 0.01 vs. control) (Fig. 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.

We occasionally observed full fusion of leptin-EGFP vesicles under GPI stimulation (Fig. 4A) and full fusion of resistin-EGFP vesicles under forskolin stimulation (Fig. 4B). As previously reported (Bai, 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, (2) accompanying increase of fluorescent intensity in the peripheral region due to diffusion of released molecules, and (3) final disappearance of the vesicle. 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 (Fig. 4C) and resistin-EGFP (Fig. 4D) are consistent with their effects on trafficking of leptin- and resistin- vesicles (Fig. 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.

**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. 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. 5a and b present the statistics (mean +/- 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 is intimately related to the actual secretion of these adipokines.

Consistent with the previous study (Levy and Stevens 2001), Fig. 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. 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 cAMP/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 cAMP/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. 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; Xue et al. 2009a). 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. 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 secretion
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. 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. 6e) was undertaken in
the time scale of min while the secretion of adipokines was measured in 2 h (Fig. 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. As a control
experiment, application of calcium ionophore calcimycin resulted in sharp increase in
[Ca$^{2+}$]$_i$ (Fig. 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.
Discussion

It has been well-documented that leptin and resistin are adipocyte-derived polypeptide hormones implicated critically in metabolic homeostasis (Lago et al. 2009; 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+}]; and 5) Interplays exist between these two adipokines. Specifically, they are all subject to regulations by insulin/glycolytic substrates, cAMP 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; Roh et al. 2000), 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; Roh et al. 2000), or lipoprotein lipase (Roh et al. 2001), or adiponectin (another adipokine) (Xie, et al. 2008). 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. 1 in Supplementary data). 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 cAMP/PKA.

It has been shown that insulin upgrades leptin expression (Barr et al. 1997) and downgrades resistin expression (Chen, et al. 2005; Haugen, et al. 2001; Shojima, et al. 2002). 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. 2 in Supplementary data), application of a protein synthesis
inhibitor (cycloheximide), which completely eliminated GPI and forskolin induced protein synthesis, was not able to block the GPI-enhanced leptin secretion or forskolin-enhanced resistin secretion (Fig. 3 in Supplementary data). 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 in Supplementary data). 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; Steppan et al. 2001). 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.
The role of calcium on leptin secretion has been studied previously in primary rat adipocytes (Cammisotto and Bukowiecki 2004; Levy, et al. 2000). 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$^{2+}$], 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 (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 cAMP/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.

**Declaration of interest:**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this scientific work.

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Reference


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**Figure Legends**

**Fig. 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 colocalization (right, merge of left and middle). (B) Distribution of overexpressed Rab5-GFP (left) and immunostained leptin (middle, Atto 647 secondary antibody), and their colocalization (right). (C) Distribution of overexpressed Rab5-GFP (left) and immunostained resistin (middle, Atto 647 secondary antibody), and their colocalization (right). (D) Distribution of overexpressed Glut4-GFP (left) and immunostained leptin (middle, Atto 647 secondary antibody), and their colocalization (right). (E) Distribution of overexpressed Glut4-GFP (left) and immunostained resistin (middle, Atto 647 secondary antibody), and their colocalization (right).

**Fig. 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 green dots. Typical motion trajectories of a subplasmalemmal leptin (A) and a resistin (B) vesicle are depicted on the right. The scale bars = 200 nm.
Fig. 3. Trafficking of leptin (A) and resistin (B) vesicles. 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).

Fig. 4. Leptin-EGFP and resistin-EGFP molecules are packed into fusion-competent vesicles. A. and B. Sequential images of full fusion event (top) of a leptin-EGFP vesicle (A) and a resistin-EGFP vesicle (B). The scale bars = 1 µm. The curves in the bottom represent the change of the mean fluorescence intensity over time within the region < 0.5 µm away from the vesicle center (solid line) and the change of fluorescence intensity within the annular region > 0.5 and < 0.65 µm away from the vesicle center (dotted line). Full vesicle fusion is characterized by simultaneous transient increase in these two curves due to rapid release of the fluorescent molecules and their diffusion to the peripheral region (Bai, et al. 2007). C. and D. Western blots of leptin-EGFP (C) and resistin-EGFP
(D) secretion under control condition, or in presence of 10 µM forskolin (F), GPI. 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 +/- 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.

Fig. 5. Western blots of leptin (A) and resistin (B) secretion under control condition (Con), or in presence of 10 µM forskolin (F), 10 µM forskolin plus GPI (GPI+F), 0.1 µM 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 +/- 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.

Fig. 6. Calcium dependence of leptin and resistin secretion. A. B. C and D. Example western blots (top) and statistics (bottom: mean +/- 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 μM) 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.
Fig. 1
Fig. 4
Fig. 5
Fig. 6