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The Crosstalks Between Adipokines and Catecholamines

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Abstract

Adipocytes, which secrete a spectrum of adipokines, play an integral role in metabolism via communications with other endocrine cells. In the present work, we have studied the interplays between adipokines and catecholamines, using 3T3-L1 adipocytes and PC12 cells as the cell models and an integrative experimental platform. We demonstrate that all catecholamines inhibit vesicle trafficking and secretion of leptin and resistin through β -adrenergic receptors, while leptin and resistin enhance the vesicle trafficking and secretion of catecholamines through PKC, PKA, MAPK kinase and Ca^{2+} dependent pathways. The crosstalks between adipokines and catecholamines were further corroborated by co-culturing 3T3-L1 adipocytes and PC12 cells. Our findings highlight the importance of adipo-adrenal axis in energy metabolism and the intricate interactions between metabolic hormones.

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; Rosen and Spiegelman, 2006). These adipokines regulate various aspects of metabolism (e.g., lipolysis and gluconeogenesis) in collaboration with other metabolic hormones, notably insulin secreted from pancreatic beta 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 the present study, using adipocytes differentiated from mouse 3T3-L1 fibroblasts and PC12 cells derived from rat chromaffin cells as the cell models, we sought to investigate the crosstalks 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.

2. Materials and Methods

2.1. Cell culture

3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD, USA) were initially maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% (v/v) bovine calf serum (Gibco) and 1% penicillin-streptomycin. To induce differentiation of 3T3-L1 fibroblasts into adipocytes, 2 days after reaching confluence (day 0), cells were cultured in DMEM containing 10% fetal bovine serum (FBS; Gibco), 10 µg/ml insulin, 0.5 mM isobutyl-1-methyl xanthine and 1 µM dexamethasone for 2 days, and then in DMEM with 10% FBS and 10 µg/ml insulin for another 2 days, followed by maintaining the cells in DMEM containing 10% FBS alone (Shao and Lazar, 1997). Adipocytes differentiation was confirmed by Oil-red-O staining and visual appearance of intracellular lipid droplets. PC12 cells (ATCC) were cultured in RPMI-1640 medium (Gibco) supplemented with 10% horse serum, 5% FBS, and 1% penicillin-streptomycin. Both 3T3-L1 and PC cells were maintained at 3

37°C under a humidified atmosphere of 5% CO₂ plus 95% air. Experiments were always conducted in parallel on the same batch of cells. Unless otherwise stated, all chemicals were purchased from Sigma (Sigma-Aldrich, Saint Louis, MO, USA).

Transwell indirect-contact coculture system (Corning Inc., Corning, NY, USA) was used to co-culture 3T3-L1 adipocytes and PC12 cells. 3T3-L1 pre-adipocytes and PC12 cells were cultured separately on either 6 well-plates or transwell inserts with porous polyester membrane (0.4 µm). Nine–10 days after initiation of adipocytes differentiation, the inserts and wells containing different cell types were assembled, and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. In the parallel control experiments, PC12 cells or 3T3-L1 adipocytes were separately cultured with the same protocol.

2.2. Plasmids and Transfections

The plasmid encoding for murine leptin cDNA was kindly provided by Professor Jan Tavernier (Ghent University, Belgium). The cDNA of murine resistin in the pCMV6 vector and the vector pEGFP-N1 were purchased from OriGene Technologies (Rockville, MD, USA) and Clontech (Mountain View, CA, USA), respectively. The plasmids of leptin-EGFP and resistin-EGFP were constructed by subcloning the leptin- or resistin- cDNA into the vector pEGFP-N1 at the N-terminus of EGFP between the Nhe I and AgeI sites (Ye et al., 2010). The plasmids were purified using Purelink HiPure miniprep kit (Invitrogen, Carlsbad, CA, USA) and verified by DNA sequencing. NPY-EGFP (neuropeptide Y tagged with enhanced green fluorescence protein) plasmid was a kind gift from Professor Wolf Almers (Vollum Institute, Oregon Health Sciences University).

Six days after initiation of differentiation, 3T3-L1 adipocytes planted on poly-L-lysine (Sigma) coated glass-coverslips were transfected with 3 µg of leptin-EGFP or resistin-EGFP plasmids using FuGENE6 Transfection Reagent (Roche, Indianapolis, IN, USA), followed by another 4-5 days culturing to attain full differentiation before imaging experiments. PC12 cells planted on the poly-L-lysine coated coverslips were transfected with 1-2 µg of NPY-EGFP plasmid using FuGENE6, 1-2 days before the imaging experiments.

2.3. Western blot analysis of adipokine secretion

3T3-L1 pre-adipocytes initially seeded in 75-cm² flasks grew till confluence and were differentiated as described above. Ten-11 days after the initiation of differentiation, fully differentiated adipocytes were washed with PBS for three times, and then incubated in serum free medium with or without testing chemicals for 120-min at 37°C. The medium were then harvested, and stored at -20°C after being concentrated using AmiconUltra centrifugal filter units (Millipore, Billerica, MA, USA). After washing the cells with ice-cold PBS for three times, a cell lysate was generated using RIPA lysis buffer containing protease inhibitors (Santa Cruz Biotech, Santa Cruz, CA, USA). Proteins concentrations in the harvested culture medium or the cell lysate were determined using a protein assay kit (Bio-Rad Lab, San Diego, CA, USA) based on Bradford method. Protein samples were separated on the 10-12% SDS-polyacrylamide gels using a Mini-Protean Tetra cell, and then transblotted onto the nitrocellulose membrane using a Mini-Transblot apparatus at 4°C (Bio-Rad Lab, Hercules, CA, USA). Membrane was then blocked for 2 hr with 5% skimmed fat milk in Tris-buffered saline-Tween (TBST) buffer (10 mM Tris, 150 mM NaCl and 0.05% Tween-20, pH7.4). Membrane was subsequently incubated for 12 hr at room temperature with primary antibodies such as rabbit anti-leptin (Sigma), rabbit anti-resistin (Biovision, Mountain View, CA, USA), rabbit anti-EGFP (Biovision), or rabbit anti-GAPDH (Sigma) antibodies (1:200-400) in TBST, followed by washing for 3 times with TBST buffer. The membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:5000; Thermo Scientific, Rockford, IL, USA) for 6-8 hr at room temperature. The protein bands were detected with CN/DAB detection kit (Thermo Scientific), and quantified using Bio-Rad's Densitometer with QualityOne software. Immunoblots of leptin, resistin and GAPDH were obtained using the same sample sets. GAPDH served as a loading control.

2.4. Enzyme-linked immunosorbent assay (ELISA) of catecholamine secretion

PC12 cells were grown in the 25 cm² flasks (3 x 10⁶ cells / flask) as described above till confluence. After washing with phosphate buffer saline, cells were incubated in serum and phenol-red free RPMI-1640 medium (Sigma) without or with testing chemicals for 1 hr at 37°C. The culture media were collected with addition of 1 mM EDTA and 4 mM sodium metabisulfite to prevent degradation of catecholamines, and frozen immediately at -80°C. Epinephrine

concentration in collected culture medium was determined using Epinephrine ELISA kit (Immuno-Biological Lab, Minneapolis, MN, USA).

2.5. Total internal reflection fluorescence microscopy (TIRFM)

TIRFM experiments were performed using a Zeiss Axiovert-200M inverted microscope system (Carl Zeiss, Göttingen, Germany) equipped with a 100x 1.45 NA TIRF objective lens. The glass-coverslip (refractive index=1.52) with 3T3-L1 adipocytes transfected with leptin-EGFP or resistin-EGFP or PC12 cells transfected with NPY-EGFP, was mounted in an imaging chamber filled with the bath-solution (150 mM NaCl, 5.4 mM KCL, 2 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, 10 mM HEPES, pH7.4). The laser beam (488 nm) was incident on the coverslip at 68-70° from normal. The penetration depth of the evanescent field was calculated to be about 100 nm. The emission of EGFP was collected at 520 nm. Using MetaMorph 6.3 (Molecular Devices, Downingtown, PA, USA), time-lapse digital images were acquired from a single cell by a CCD camera with exposure times of 25 ms and 2 Hz sampling frequency for 2-min. Large dense core vesicles specifically labeled by overexpressing NPY-EGFP in PC12 cells, and leptin vesicles labeled by overexpressing leptin-EGFP or resistin vesicles labeled by overexpressing resistin-EGFP in 3T3-L1 adipocytes, were individually tracked using ImageJ (National institute of Health, <http://rsb.info.nih.gov/ij/>).

2.6. Amperometry recording and analysis

Amperometry recording was conducted by an EPC-10 double patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) using a 5-μm carbon fiber microelectrode (ALA Scientific Instruments, Westbury, NY, USA). PC12 cells were incubated in the bath solution (same as used in TIRM experiments) without or with 50 nM leptin or 50 nM resistin (Biovision) for 30-min before recording. For each recording, the tip of the carbon fiber was freshly cut and gently positioned onto the cell membrane. While the cell was superfused with high K⁺ solution (40 mM NaCl, 105 mM KCl, 1 mM MgCl₂, 6 mM CaCl₂, 10 mM HEPES, pH 7.4) using an application pipette in order to trigger vesicular secretion (exocytosis), the amperometric signals was recorded for 2-min with 4 kHz sampling rate and 1 kHz filtering frequency. The amperometric signals were analyzed by a Igor program, Amperometric Spike Analysis 8.15, developed by Dr.

Eugene Mosharov (Department of Neurology, Columbia University). Spikes with amplitudes less than 2 pA (5 times of the background noise) were considered as noise and excluded for analysis.

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

PC12 cells grown on the poly-L-lysine coated glass-coverslips were loaded with 5 μ M Fura-2-AM (Molecular Probes, Eugene, OR, USA) for 40-60 min in culture medium at 37°C. The cells were then rinsed and rested for 30-min in the bath solution to allow de-esterification and retention of Fura-2-AM. Cells were incubated in the bath solution as defined above without or with 50 nM leptin or 50 nM resistin for 30-min before recording. Intracellular calcium concentration ($[Ca^{2+}]_i$) was reported as the ratio of emissions (F340/F380) at 510 nm resulting from the excitation wavelengths of 340 nm and 380 nm. Fluorescence signals in response to 2-min perfusion high K^+ solution were recorded from single cell using a photometry system (TILL Photonics GmbH, Gräfelfing, Germany).

2.8. Statistical analysis

Results are reported as mean $\pm$ SEM, and the statistical significance was analyzed by Student's unpaired t-test or the Mann-Whitney rank sum test for skewedly distributed vesicle velocity (Ye et al., 2010). $p < 0.05$, $p < 0.01$, $p < 0.001$ were considered statistically significant.

3. Results

3.1. 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. 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.

Both α - and β - adrenergic receptors are expressed in white adipocytes (Fain and Garcia-Sainz, 1983). The suppressive effect of epinephrine (Fig. 2A) and norepinephrine (not shown) on adipokine secretion was essentially relieved by co-application of propranolol (10 μ M, β -

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adrenergic receptor antagonist), but not affected by phentolamine (10 μ M, α -adrenergic receptor antagonist). The inhibitory effect of dopamine (Fig. 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.

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

In the previous study (Ye et al., 2010), 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 (Chen and Gillis, 2000; Xue et al., 2009a), 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, we showed previously that (Ye et al., 2010), just like other secretory vesicles in neurons or endocrine cells (Holz and Axelrod, 2008; Zhang et al., 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.

Here, individual leptin-EGFP labeled vesicles (Fig. 3A) and resistin-EGFP labeled vesicles (Fig. 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. 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). 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. 3 and 4). All these observations are expectedly consistent with the western blot analyses of actual adipokine secretion (Fig. 1 and 2).

We have demonstrated previously that 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 (Ye et al., 2010). Here, we show that secretion of leptin-EGFP and resistin-EGFP was also inhibited by catecholamines (Supplemental Fig. 1).

3.3. 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. 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) dependent ways (Takekoshi et al., 1999; Takekoshi et al., 2001b; Shibuya et al., 2002). 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. 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.

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. 5).

3.4. 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; Zhang et al., 2008; Xue et al., 2009b; Zhang et al., 2009). 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. 6A). Indeed, as revealed by TIRFM, lateral as well as vertical trafficking of these vesicles was largely enhanced by 50 nM of leptin (Fig. 6B) or resistin treatment (Fig. 6C). Also in agreement with the ELISA results on actual epinephrine secretion (Fig. 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.

3.5. Effects of leptin and resistin on triggered vesicle secretion of catecholamines

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. 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

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oxidization of catecholamine molecules on the voltage-biased CFM electrode (Xue et al., 2009a). As demonstrated in Fig. 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. 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. 5).

3.6. Coculturing 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 Transwell 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. 8A). As shown in Fig. 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. 1 and 5. Peculiarly, 3-4 days co-culturing leads to increase of leptin and resistin secretion and more significant increase of catecholamine secretion (Supplemental Fig. 2). 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 (Supplemental Fig. 2). 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.

4. 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 crosstalks 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 crosstalks 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. 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 preadipocytes instead of mature human adipocytes (Savage et al., 2001; Janke et al., 2002).

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., 2007; 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., 1997). 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., 2001). Such inhibition may also be accounted by the reduced vesicle trafficking (Fig. 3).

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 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 (Supplemental Fig. 3). This adds another dimension in the adipo-adrenal axis.

Although leptin and resistin are differentially regulated and sometimes function oppositely (Pui et al., 2009; Ye et al., 2010), 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. 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. 6 and 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., 2002; Xue et al., 2009a).

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 (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. This could be due to downregulation of adrenergic receptors (Bottner et al., 1999) and promoted hypertrophy of adipocytes. And neuropeptide Y (NPY) secreted by chromaffin and PC12 cells is 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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Fig. 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.

Fig. 2. Catecholamines inhibit leptin and resistin secretion from 3T3-L1 adipocytes through the β -adrenergic receptors. **(A)** Top panel shows the representative immunoblots of leptin (left) or resistin (right) released to the culture medium in 2 hr, without (control or Con) or in presence of epinephrine (E, 1 μ M) or epinephrine plus phentolamine (10 μ M, 30-min pretreatment) or epinephrine plus propranolol (10 μ M, 30-min pretreatment). **(B)** Top panel shows the representative immunoblots of leptin (left) or resistin (right) released to the culture medium in 2 hr, without (control or Con) or in the presence of dopamine (DA, 1 μ M) or dopamine plus phentolamine (10 μ M, 30-min pretreatment) or dopamine plus propranolol (10 μ M, 30 min-pretreatment) or dopamine plus haloperidol (10 μ M, 30-min pretreatment) or dopamine plus

SCH-23390 (10 μ M, 30-min pretreatment). **In (A) and (B)**, GAPDH serves as a loading control. The lower panel shows the statistics (mean $\pm$ SEM, n = 5-7) of optical density of the blots normalized to the density of GAPDH blots in arbitrary unit (a.u.). Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

Fig. 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 ~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.

Fig. 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 (B). 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.

Fig. 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.

Fig. 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 μ M, 1 hr pretreatment). The values represent mean $\pm$ SEM (8 cells from 3-4 independent experiments and ~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.

Fig. 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 preincubation 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.

Fig. 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.