Insulin-stimulated leptin secretion requires calcium and PI3K/Akt activation

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Numerous studies have focused on the regulation of leptin signalling and the functions of leptin in energy homeostasis; however, little is known about how leptin secretion is regulated. In the present study we studied leptin storage and secretion regulation in 3T3-L1 and primary adipocytes. Leptin is stored in membrane-bound vesicles that are localized predominantly in the ER (endoplasmic reticulum) and close to the plasma membrane of both 3T3-L1 and primary adipocytes. Insulin increases leptin secretion as early as 15 min without affecting the leptin mRNA level. Interestingly, treatment with the protein synthesis inhibitor cycloheximide and the ER–Golgi trafficking blocker Brefeldin A inhibit both basal and ISLS (insulin-stimulated leptin secretion), suggesting that insulin stimulates leptin secretion by up-regulating leptin synthesis and that leptin-containing vesicles go through the ER–Golgi route. The PI3K (phosphoinositide 3-kinase)/Akt, but not MAPK (mitogen-activated protein kinase), pathway is involved in ISLS in vitro and in vivo. Although Ca2+ triggers synaptic vesicle and secretory granule exocytosis, Ca2+ influx alone is not sufficient to induce leptin secretion. Remarkably, Ca2+ is required for ISLS possibly due to its involvement in insulin-stimulated Akt phosphorylation. We conclude that insulin stimulates leptin release through the PI3K/Akt pathway and that Ca2+ is required for robust Akt phosphorylation and leptin secretion.

Key words: Akt, Ca2+, insulin, leptin, phosphoinositide 3-kinase (PI3K), primary adipocyte, secretion.

INTRODUCTION

WAT (white adipose tissue) has gained significant scientific interest as the increasing prevalence of obesity has become a burgeoning public health problem. What was once considered as a storage depot is now recognized as a vital integrator of homoeostatic processes, co-ordinated mainly through the synthesis and release of peptide hormones by adipocytes [1]. One of the main peptides released from WAT is the adipokine leptin, a 16-kDa protein that is encoded by the LEP (also known as OB) gene [2]. In addition to the many functions of leptin in peripheral systems, such as the muscle and liver, the central nervous system remains one of the most important targets of leptin [3]. Upon its secretion from adipocytes, leptin circulates and binds to its receptor in peripheral organs, including the skeletal muscle and the central nervous system, such as the in hypothalamus to regulate energy homoeostasis by increasing energy expenditure and reducing food intake [4].

To date, many studies have reported on the regulation of leptin signalling and the function of leptin in a variety of target tissues; however, little is known about the regulation of leptin secretion from WAT. Several lines of evidence have suggested that leptin secretion from WAT may be regulated (see [5] and references cited therein): (i) after fasting, the plasma leptin level decreases [6]; (ii) after a meal, plasma leptin levels rapidly increase despite no change in WAT mass [6]; (iii) insulin infusion increases circulating leptin concentrations in human subjects [7]; and (iv) blockade of glucose transport or glycolysis inhibits leptin expression and secretion in isolated adipocytes [8]. As dysregulated leptin release from adipocytes results in an imbalance in energy homoeostasis, it is critical to elucidate the mechanism and regulation of leptin secretion [9]. It is unclear whether dysregulated leptin secretion from WAT plays a direct role in leptin resistance of obese patients. In addition, leptin administration is able to prevent hyperglycaemia in a Type 1 diabetes mouse model, suggesting that proper control of leptin secretion is crucial for normal physiology [10]. Thus it is of significant importance to understand how leptin secretion is regulated.

Insulin is one of the most important stimulators of leptin production and secretion [11–13], and leptin synthesis and secretion appear to be Ca2+-dependent in 3T3-L1 cells [14]. However, none of these previous studies have revealed a mechanism for the understanding of leptin storage and the role of Ca2+ in ISLS (insulin-stimulated leptin secretion). In the present study, we seek to elucidate the precise mechanism that synergizes leptin vesicle localization, Ca2+-dependence and ISLS in white adipocytes. We also examine the secretory pathway and the signalling events that underlie ISLS. We demonstrate that insulin is packaged into membrane-bound secretory vesicles and goes through the ER (endoplasmic reticulum)–Golgi secretory pathway. Insulin stimulates leptin secretion through PI3K (phosphoinositide 3-kinase)/Akt pathway. Although Ca2+ influx is not sufficient to trigger acute leptin secretion, it is essential for leptin secretion by affecting Akt phosphorylation thereby linking Ca2+ with insulin signalling in leptin secretion from white adipocytes.

Abbreviations: BAPTA/AM, 1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid tetrasodiumtetraacetate (acetoxymethyl ester); BFA, Brefeldin A; CD, chow diet; CHX, cycloheximide; DIO, diet-induced-obesity; EEA1, early endosome antigen 1; EGTA/AM, EGTA acetoxyethyl ester; ER, endoplasmic reticulum; GLUT4, glucose transporter 4; HDM, high-density microsome; HFD, high-fat diet; ISLS, insulin-stimulated leptin secretion; LDM, low-density microsome; LEP, leptin gene; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PM, plasma membrane; TIRF, total internal reflection fluorescence; WAT, white adipose tissue.

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MATERIALS AND METHODS

Animal welfare

Male 7–12-week-old C57BL/6 mice were obtained from the Biological Resource Center of the Agency for Science, Technology and Research (A*STAR) and all experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the A*STAR. For study of DIO (diet-induced-obesity) mice, C57BL/6 male mice were divided into two groups at 7–8 weeks old, with one group receiving a HFD (high-fat diet; Research Diet D12492; 60 % kcal% fat) and the other receiving a CD (chow diet; Research Diet D12450B; 10 % kcal% fat) for 16 weeks. Mice were group-housed with five mice in each cage and the temperature of the animal holding room was maintained at 22–25 °C.

Cell culture

3T3-L1 cells were obtained from the A.T.C.C. (Manassas, VA, U.S.A.) and cultured and differentiated as described previously [15]. Primary adipocytes were isolated from mouse epididymal fat pads as previously described [16] with slight modifications. The pLenti-Leptin-Venus and pLenti-Venus constructs were used to generate 3T3-L1 stable cell lines expressing leptin–Venus (3T3-LV) and Venus (3T3-V) respectively.

RNA extraction and real-time PCR

Total RNA extraction and quantitative real-time PCR were performed essentially as previously described [17]. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the endogenous reference.

Subcellular fractionation, immunocytochemistry and immunoblotting

Subcellular fractionation and trypsin protection assays were performed as described previously [18,19]. The 3T3-LV cells were washed with PBS, fixed in PBS containing 4 % paraformaldehyde for 10 min, permeabilized in PBS containing 0.2 % saponin for 5 min and blocked in blocking buffer (3 % BSA and 3 % goat anti-serum in PBS) for 1 h at room temperature (22–25 °C). The cells were then probed using indicated antibodies and fluorescence-conjugated secondary antibodies (Life Technologies). The coverslips were mounted on to slides and observed by confocal microscopy. For immunoblotting, adipocytes were washed with BSA-free KRH buffer (Kreb-Ringer Hepes; 130 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM glucose and 20 mM Hepes, pH 7.4) and lysed in whole cell lysis buffer. Proteins were resolved by SDS/PAGE (10 % gel), transferred on to PVDF membranes and probed with antibodies according to the manufacturer’s instructions.

Fluorescence microscopy imaging

TIRF (total internal reflection fluorescence) microscopy imaging was performed using an inverted microscope system equipped with a ×100 1.45 NA (numerical aperture) objective (Nikon). For intracellular Ca²⁺ measurements, freshly isolated mouse white adipocytes were loaded with 3 μM fura 2. Images were collected at 10 s intervals, and fluorescence signals from individual cells were measured as a function of time using MetaFluor software (Molecular Devices).

Statistical analysis and experimental details

All leptin concentrations were normalized to the respective basal leptin concentration of adipocytes. Statistical comparisons were performed by ANOVA followed by Tukey’s post-hoc test, or two-tailed independent Student’s t test. The significance limit was set at P < 0.05. Further details of the experimental procedures, chemicals and reagents are given in the Supplemental Online Data (http://www.biochemj.org/bj/458/bj4580491add.htm).

RESULTS

Leptin is packaged into membrane-bound secretory vesicles

To determine the secretory pathway that underpins leptin secretion, we generated 3T3-L1 stable cell lines expressing either leptin–Venus fusion protein (3T3-LV) or Venus alone (3T3-V) (Supplementary Figures S1A and S1B at http://www.biochemj.org/bj/458/bj4580491add.htm). Although the 3T3-LV cells had a high basal secretion of leptin, insulin was able to further stimulate leptin secretion (Supplementary Figure S1C), thus confirming that these cells behave as bona fide leptin-secreting cells. Using TIRF microscopy, the fusion protein leptin–Venus was observed to be localized close to the PM (plasma membrane) with a clear punctate distribution, suggesting that leptin is packaged into vesicular structures (Figure 1A). To confirm the vesicular packaging of leptin, we performed a trypsin protection assay in both 3T3-LV and primary adipocytes. As expected, leptin was detected in lysates treated with trypsin alone, whereas cytosolic and transmembrane proteins, e.g. actin and GLUT4 (glucose transporter 4), were digested and undetectable (Figure 1B). In contrast, leptin was completely digested by trypsin when lipid bilayers were solubilized with Triton X-100, indicating that leptin was packaged inside membrane-bound vesicles in both 3T3-LV and primary adipocytes.

We next examined leptin localization by confocal microscopy and found that leptin co-localized with the ER marker motif KDEL, but to a lesser extent with calnexin or the early endosome marker EEA1 (early endosome antigen 1) (Figure 1C). We did not detect co-localization of leptin with the Golgi markers syntaxin 6 or golgin 97. Leptin appeared to localize in compartments close to the PM; however, there was no significant co-localization between caveolin 1 and leptin-containing vesicles. We examined further leptin localization by subcellular fractionation of 3T3-LV and primary adipocytes. Leptin was found mainly in the HDM (high-density microsome), and to a lesser extent the PM and primary adipocytes.

ISLS requires continued leptin synthesis and trafficking

We sought to uncover the regulation sites of ISLS by using primary adipocytes. We examined leptin secretion within the first 2 h of insulin stimulation. Insulin steadily increased leptin
Regulation of leptin secretion by insulin and Ca²⁺

Figure 1  Leptin is stored in membrane-bound vesicles in adipocytes

(A) Fluorescence images showing distribution of leptin–Venus or Venus alone in stable 3T3-L1 adipocytes expressing leptin–Venus (3T3-LV) or Venus (3T3-V) under epifluorescence (EPI) or TIRF microscopy. Scale bar, 5 μm. (B) Cell lysates of 3T3-LV and primary adipocytes were incubated with combination of trypsin and Triton X-100 (TX-100), as indicated, at 4°C followed by Western blotting using antibodies against GLUT4, leptin and actin. (C) 3T3-LV adipocytes were fixed, permeabilized and probed with antibodies against various organelle markers as indicated. Scale bar, 5 μm. (D) Subcellular fractionation of 3T3-LV homogenates and Western blotting using the indicated antibodies. (E) Subcellular fractionation of primary adipocyte homogenates and leptin concentration measurements by ELISA. Data were normalized to the value of the leptin level in the HDM and shown as means ± S.D.; n = 3.

secretion across the 2-h stimulation period (Figure 2A). There was no change in leptin mRNA levels in primary adipocytes (Figure 2B), suggesting that insulin promotes leptin secretion by enhancing leptin synthesis and/or trafficking in the first 2 h without affecting LEP gene transcription. We then tested the role of leptin synthesis in supporting ISLS by pre-incubating primary adipocytes with the protein synthesis inhibitor CHX (cycloheximide) before insulin stimulation. An incubation time of 4 h with CHX was sufficient to abrogate ISLS (Figure 2C). Next, we pre-treated the adipocytes with CHX for 4 h before a 2-h insulin stimulation and found that insulin failed to stimulate leptin secretion (Figure 2D), although adiponectin secretion remained intact (Supplementary Figure S2 at http://www.biochemj.org/bj/458/bj4580491add.htm), suggesting that ISLS requires continued leptin synthesis. The fact that adiponectin secretion was not affected by CHX treatment indicates that there may exist a pre-formed pool of adiponectin vesicles to support insulin-stimulated adiponectin secretion, but no such pool of leptin vesicles exists to support ISLS. We also evaluated the involvement of membrane trafficking, in particular ER–Golgi vesicle trafficking...
in ISLS by incubating primary adipocytes with BFA (Brefeldin A), an ER–Golgi vesicle trafficking inhibitor. BFA inhibited significantly basal leptin secretion, suggesting that leptin vesicles move through the ER–Golgi secretory pathway (Figure 2E). Furthermore, BFA blocked ISLS, supporting the notion that there are no preformed leptin vesicles awaiting insulin stimulation. Together, these results show that ISLS depends on continued supply of leptin through new protein synthesis and efficient ER–Golgi vesicle trafficking.

Activation of PI3K/Akt, but not MAPK (mitogen-activated protein kinase) is required in ISLS

To examine the involvement of the canonical insulin/PI3K/Akt pathway in adipocyte leptin secretion, we measured leptin secretion in insulin-resistant DIO mice. Age-matched male mice fed on a HFD for 16 weeks had significantly higher body mass and body fat mass than those fed on a CD (Figure 3A). The phospho-Akt level in adipocytes from HFD-fed mice was significantly lower compared with that of control mice after insulin stimulation, consistent with insulin resistance in HFD-fed mice (Figure 3B). The fasting leptin level of HFD-fed mice was approximately 10-fold higher than that of the control mice. However, upon refeeding, there was no significant increase in plasma leptin level in HFD-fed mice even after 2 h, whereas the control mice showed a significantly higher plasma leptin level (Figure 3C). Hence, leptin production and/or secretion after refeeding were impaired in HFD-fed mice. To verify the diminished systemic leptin levels in re-fed HFD-fed mice, we isolated primary adipocytes from HFD-fed mice and control mice to determine ISLS ex vivo. ISLS was significantly lower in primary adipocytes from HFD-fed mice from the 30 min time point onwards (Figure 3D). These results support the hypothesis that impaired ISLS in HFD-fed mice was dependent on PI3K/Akt activation.

We then confirmed further the essential role of PI3K/Akt in regulating ISLS in primary adipocytes. Blocking PI3K activity with wortmannin significantly inhibited ISLS without affecting basal leptin secretion (Figure 3E), consistent with a previous report [12]. Similarly, the Akt1/2 inhibitor Akti impaired significantly ISLS with no significant effect on basal leptin secretion (Figure 3F). In contrast, the MAPK inhibitor PD98059 had no effect on ISLS (Supplementary Figure S3 at http://www.biochemj.org/bj/458/bj4580491add.htm). Collectively, these findings suggest that the PI3K/Akt, but not the MAPK, pathway is required for ISLS from primary adipocytes.

We also examined the effects of short-term insulin signalling on leptin secretion by measuring ISLS from 3T3-LV cells and primary white adipocytes at several time points within the first 15 min of insulin stimulation. No statistical difference was detected at these time points between groups receiving mock, insulin and insulin plus wortmannin treatment (Supplementary

**Figure 2** ISLS requires continued leptin synthesis and efficient membrane trafficking

(A) Primary adipocytes received mock or 1 μg/ml insulin for 15, 30, 60 and 120 min. The incubation media were collected and subjected to leptin ELISA; n = 12. (B) Primary adipocytes were treated with or without 1 μg/ml insulin for 2 h and total RNA was extracted and subjected to real-time PCR; n = 12. (C) Primary adipocytes received mock or 100 μg/ml CHX for 2, 4 and 6 h. The incubation media were collected and subjected to leptin ELISA; n = 9. (D) Primary adipocytes were treated with or without 100 μg/ml CHX for 4 h followed by 1 μg/ml insulin stimulation for 2 h. The incubation media were collected and subjected to leptin ELISA; n = 7. (E) Primary adipocytes were treated with 5 μg/ml BFA or vehicle for 30 min followed by 1 μg/ml insulin stimulation for 2 h. The incubation media were collected and subjected to leptin ELISA; n = 6. Results are means ± S.E.M. *P < 0.05 and **P < 0.01.
Ca^{2+} is necessary for ISLS, but not sufficient to trigger leptin secretion

Considering the importance of Ca^{2+} in triggering regulated exocytosis in neurons and endocrine cells [20], we next determined the role of Ca^{2+} in ISLS from adipocytes. When intracellular Ca^{2+} was chelated with EGTA/AM (EGTA acetoxy-methyl ester) or BAPTA/AM [1,2-bis-(o-aminophenoxy)ethane-
N,N',N'-tetra-acetic acid tetrakis(acetoxymethyl ester)], basal leptin secretion was reduced significantly. More importantly, ISLS was completely blocked in the absence of Ca^{2+} (Figures 4A and 4B), suggesting that Ca^{2+} is required for ISLS. We then tested whether an elevated Ca^{2+} level was sufficient to trigger leptin secretion by treating primary adipocytes with ionomycin. Unlike in synaptic vesicle and secretory granule exocytosis, Ca^{2+} influx induced by ionomycin had no significant effect on leptin secretion in primary adipocytes (Figures 4C–4E), indicating that Ca^{2+} influx was not sufficient to trigger leptin secretion. Consistent with this notion, insulin did not cause Ca^{2+} level changes as measured by fura 2 in primary adipocytes (Figure 4C). Collectively, these results suggest that basal Ca^{2+} level is necessary to support ISLS, but not sufficient to trigger leptin secretion.

As depletion of intracellular Ca^{2+} had no inhibitory effect on the proximal insulin signalling action, such as insulin receptor autophosphorylation, IRS-1 (insulin receptor substrate-1)
tyrosine phosphorylation or activation of PI3K [14,21], we decided to test directly Akt phosphorylation in primary adipocytes in the absence of Ca\(^{2+}\). We depleted intracellular Ca\(^{2+}\) by treating the cells with membrane-permeable EGTA/AM or BAPTA/AM. Remarkably, in the absence of Ca\(^{2+}\), insulin-induced Akt phosphorylation was significantly attenuated in isolated adipocytes (Figure 4F), consistent with a previous report using 3T3-L1 cells [11]. Together, both insulin signalling and Ca\(^{2+}\) are required for the activation of Akt and stimulation of the subsequent leptin secretion from adipocytes.

**DISCUSSION**

In the present study, we demonstrate that leptin is packaged into membrane-bound vesicles and that ISLS requires Ca\(^{2+}\) for robust Akt phosphorylation. The putative mechanism is supported by our observations reported herein and defines regulated leptin secretion by adipocytes from synthesis to release. Deranged leptin secretion from adipocytes is detrimental to whole-body physiology and understanding of leptin secretion may be useful in the treatment of obesity and metabolic diseases.

The results of the present study suggest strongly that ISLS is through a tonic secretory pathway and not a phasic-regulated pathway, such as tightly time-controlled Ca\(^{2+}\)-triggered neurotransmitter and hormone secretion [20,22], on the basis of the following lines of evidence. First, time-dependent ISLS was almost parallel to that of basal leptin secretion and there was no sudden rapid increase in leptin concentration upon insulin treatment (Figure 2A). Therefore the source of stimulated leptin secretion is unlikely to be from pre-formed pools of leptin, but rather increased secretion upon leptin synthesis. It remains possible that insulin may increase the rate of vesicle trafficking
along the ER–Golgi secretory pathway or at the post-Golgi membrane events. Secondly, we found that after blocking leptin synthesis and basal leptin secretion by CHX insulin failed to stimulate further leptin secretion (Figure 2D), consistent with previous findings [23–25]. Reports showing that CHX could not block ISLS were most probably due to the incomplete inhibition of the basal leptin secretion. In the present study, CHX completely inhibits the basal leptin secretion only after 4 h of pre-treatment. It is worth noting that effects of CHX can also be reversed by removing it from the incubation medium. Therefore there remains a possibility of incomplete inhibition of protein synthesis by CHX before and during insulin treatment in these conflicting reports. Thirdly, we show that ISLS was inhibited completely after blocking the ER–Golgi trafficking with BFA (Figure 2E), suggesting that there are no mature leptin-containing vesicles stored outside the ER and Golgi in adipocytes. Taken together, the results of the present study demonstrate that ISLS is achieved through the enhanced synthesis of leptin. These observations therefore corroborate earlier reports that suggested a constitutive leptin-release pathway [23–25], but contradict those that suggested a regulated secretory pathway for ISLS [12,26].

To date, the nature of the downstream insulin signalling pathways involved in ISLS lacked consensus. It has been suggested by several studies that the PI3K pathway is involved in leptin secretion, because the PI3K inhibitor LY294002 could attenuate ISLS [12,27]. However, Zeigerer et al. [28] reported that wortmannin, another PI3K inhibitor, did not inhibit ISLS in 3T3-L1 adipocytes. The observed differences may stem from the different cell types used, 3T3-L1 compared with primary adipocytes. We found that wortmannin treatment significantly blocked ISLS in primary adipocytes (Figure 3E). We demonstrated further that the activity of Akt is important for ISLS (Figure 3F). More importantly, leptin secretion induced by refeeding after fasting was also impaired significantly in insulin-resistant DIO mice. ISLS in the primary adipocytes isolated from the DIO mice was also decreased compared with non-obese mice (Figure 3). The PI3K/Akt pathway in these mice was impaired as phosphorylation of Akt in WAT was decreased dramatically (Figure 3B), suggesting that PI3K/Akt is a key regulator that controls ISLS.

Ca2+ triggers membrane trafficking is of central significance for synaptic vesicle exocytosis and insulin secretion in pancreatic β-cells [20,29]. We thus also addressed the precise role of Ca2+ in leptin secretion in the present study. Previous studies have shown that Ca2+ was important for insulin-stimulated, but not basal, leptin secretion [16,30]. In contrast, the evidence of the present study suggests that basal leptin secretion was decreased and ISLS was completely blocked in the absence of Ca2+. Ca2+ may be involved in the trafficking and membrane-fusion process of leptin-containing vesicles, thus depleting Ca2+ has a major effect on basal leptin secretion. Unlike the triggering role of Ca2+ in neurotransmitter and polypeptide hormone secretion [22,31], intracellular Ca2+ rise in the presence of a Ca2+ ionophore is not sufficient to effect leptin secretion directly. Furthermore, insulin alone was insufficient to induce a rise in intracellular Ca2+ concentration, consistent with the notion that Ca2+ is not a triggering signal, but rather a regulator of ISLS. In the present study, we show that ISLS is dependent on Ca2+ and that Ca2+ is required for robust phosphorylation of Akt. Since the phosphorylation of Akt depends on Ca2+, a mechanism that allows for a cross-talk between Ca2+ influx and downstream insulin signalling, must exist in isolated white adipocytes. Ca2+ may affect Akt phosphorylation through calmodulin, as W13, a calmodulin inhibitor, decreased Akt phosphorylation in 3T3-L1 cells [21]. Another calmodulin inhibitor, W-7, was shown to block the Akt phosphorylation in a breast cancer cell line [32]. In addition, a loss of intracellular Ca2+ may also affect the translocation of Akt to the cell membrane thereby preventing its phosphorylation [14]. Whether or not these mechanisms occur in the adipocytes remains a subject for future investigation.

As shown in the present study, ISLS depends on protein synthesis and the PI3K/Akt pathway may mediate the effect of insulin on leptin secretion by increasing leptin synthesis through activation of the mTOR (mammalian target of rapamycin) pathway. PI3K/Akt/mTOR is a well-established pathway regulating protein synthesis [33]. Consistently, inhibition of mTOR activity was reported to result in decreased ISLS [12]. Therefore mTOR could be a key downstream target of Akt in regulating leptin secretion. Insulin may also potentiate leptin secretion through other mechanisms, such as through the regulation of leptin secretory vesicle trafficking and membrane fusion. We examined this scenario by measuring leptin secretion within 15 min after insulin application with or without the PI3K inhibitor wortmannin, and observed no suppression of basal leptin release by wortmannin and no obvious effects on ISLS (Supplementary Figure S4), thus it is unlikely that the PI3K/Akt pathway is involved directly in the leptin-containing vesicle trafficking or membrane fusion. In contrast, it was reported previously that insulin-stimulated actin polymerization is important for GLUT4 vesicle–PM fusion in adipocytes [34]. Therefore adipocytes may have evolved to utilize multiple distinct pathways to regulate the exocytosis of various adipokine-containing vesicles from GLUT4 storage vesicles. The detailed mechanisms and downstream effectors in these pathways remain to be elucidated.

AUTHOR CONTRIBUTION

Yue Wang, Wanjin Hong, Zhiping Pang and Weiping Han conceived the study and designed the experiments. Yue Wang, Yusuf Ali and Chun-Yan Lim conducted the experiments and analysed the data. Yue Wang, Zhiping Pang and Weiping Han wrote the paper. All authors discussed and approved the paper.

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SUPPLEMENTARY ONLINE DATA

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MATERIALS AND METHODS

Plasmid construction

The pLeptin-Venus plasmid was generated as follows: full-length leptin sequence was amplified by PCR and cloned into pEGFP-N1 to produce pLeptin. Site-directed mutagenesis was then performed to introduce a KpnI site between the signal sequence and leptin sequence of pLeptin, followed by insertion of a linker sequence at the newly created KpnI site to generate pLeptin-linker. The Venus-encoding sequence was amplified by PCR and inserted into the pLeptin-linker to produce pLeptin-Venus. The pVenus plasmid was made by inserting the Venus sequence into the pEGFP-N1. The leptin–Venus and Venus sequence in pLeptin-Venus and pVenus was cut out with Nhel and Hpal and subcloned into pLenti-Hiko to make the pLenti-Leptin-Venus and pLenti-Venus respectively. The primers sequences are given in Table S1.

3T3-L1 cell culture

3T3-L1 fibroblasts were obtained from the A.T.C.C. (Manassas, VA, U.S.A.) and cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 25 mM glucose and 10% FBS at 37°C with 5% CO2. For differentiation, cells were grown to confluence and culture medium was changed to DMEM supplemented with 10% FBS to culture for 2 days. At this point, referred to as day 0, 3T3-L1 cells were differentiated by an adipogenic cocktail containing 1 μg/ml insulin, 500 μM methyl-isobutyl-xanthine and 1 μM dexamethasone for 2 days. Cell culture medium was changed to the insulin medium containing 1 μg/ml insulin on day 2 and cultured for 2 additional days. On day 4, the culture medium was changed to DMEM with 10% FBS and cultured to days 8–10 with the medium changed every 2 days.

Lentivirus production and infection

Lentiviral particles expressing leptin–Venus or Venus were generated essentially as described previously [1,2]. Fresh 3T3-L1 culture medium and virus-containing medium were mixed at a 1:1 ratio and added to wild-type 3T3-L1 cells. Polybrene (8 μg/ml) was added to enhance the efficiency of viral infection. Cell culture medium was changed after 16 h and the cells showed fluorescence approximately 48 h after infection. Cells were not sorted as almost 100% of the cells were infected by observation under fluorescent microscope. 3T3-L1 cells stably expressing leptin–Venus fusion protein was designated as 3T3-LV and the cells stably expressing Venus as 3T3-V.

Primary adipocyte isolation

Adipocytes were isolated from mouse epididymal fat pads as described previously with slight modifications [3]. Briefly, mouse epididymal fat pads were collected and placed in KRH (Kreb-Ringer Hepes) buffer, pH 7.4, supplemented with 1% fatty acid-free BSA and 2.5 mM CaCl2 (1% KRH). The minced tissue was incubated in the above buffer plus 2 mg/ml collagenase type IV (Roche) at 37°C for 30–60 min with shaking at 150 rev/min. After digestion, the cells were passed through a 200 μm nylon filter (Biomed Diagnostics) and washed three times with 1% KRH buffer and aliquoted into 100–200 μl to be equilibrated at 37°C for 30 min (shaking frequency of 50 rev/min). For studies performed in the absence of extracellular Ca2+, cells were washed three times with a Ca2+-free KRH buffer containing 4% fatty acid-free BSA (4% KRH) with 5 mM BAPTA or EGTA. Adipocytes were incubated in the Ca2+-free 4% KRH buffer for 30 min followed by incubation in the presence or absence of insulin for 2 h. For the studies using Ca2+-containing KRH buffer, adipocytes were washed with 4% KRH buffer three times, pretreated with the agents to be tested for 30 min and incubated with or without insulin for 2 h. At the end of incubation, the incubation medium was collected for leptin concentration measurement by ELISA using kits with a detection limit of 0.05 ng/ml (Millipore).

TIRF microscopy imaging

TIRF microscopy imaging was carried out at 30°C using an inverted microscope system (Nikon) equipped with a ×100 1.45 NA (numerical aperture) TIRF objective. 3T3-LV and 3T3-V cells were cultured and differentiated to days 7–9. The cells were re-plated on to poly-L-lysine-coated coverslips and cultured overnight. The coverslips with cells were mounted in a custom-made chamber and incubated in 4% KRH buffer. The cells were imaged using epifluorescence and TIRF microscopy. Venus was excited by a 488 nm laser and the emission was collected with a bandwidth filter centred at 520 nm. For TIRF, 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 was ~100 nm.

Ca2+ imaging

Freshly isolated mouse adipocytes were loaded with 3 μM fura 2 for 40 min in 4% KRH buffer. Then the cells were rinsed three times with fresh 4% KRH buffer and seeded on to an ECM (extracellular matrix) gel-coated coverslip. The coverslip was placed in the perfusion chamber on the stage of an inverted microscope (Nikon Eclipse TE2000-U) and continuously perfused with 4% KRH buffer at 37°C. The emitted signal was collected using a 535DF35 band pass optical filter (Omega Optical) using a CCD (charge-couple device) camera (CoolSNAP HQ2; Photometrics). The adipocytes were perfused with 4% KRH buffer for 10 min and then stimulated with ionomycin.
Figure S1 3T3-L1 cells stably expressing leptin–Venus or Venus

(A) Schematic drawing showing the leptin–Venus fusion protein, in which Venus coding sequence was inserted into the N-terminus of leptin immediately downstream of the signal sequence (ss). (B) Cell lysates of HEK (human embryonic kidney)-293T (293T) cells transfected with empty vector (WT), pVenus (V) or pLeptin-Venus (LV), and 3T3-L1 (WT), stable 3T3-L1 cells expressing Venus (V) or leptin–Venus (LV) were analysed with antibodies against GFP or leptin; n = 3. Molecular mass is given on the left-hand side in kDa. IB, immunoblotting. (C) 3T3-V and 3T3-LV cells were differentiated into mature adipocytes and treated with or without insulin for 2 h. The culture medium was collected for leptin concentration measurement by ELISA; n = 6. Results are means ± S.D. *P < 0.05.

Figure S2 Effect of CHX treatment on adiponectin secretion

(A) Primary adipocytes receiving mock or 100 μg/ml CHX treatment for 2, 4 and 6 h. The incubation medium was collected and adiponectin levels measured by ELISA; n = 9. (B) Primary adipocytes receiving mock or CHX treatment for 4 h followed by insulin or vehicle treatment for 2 h as indicated. The incubation medium was collected and adiponectin levels measured by ELISA; n = 7. *P < 0.05 and **P < 0.01.

Figure S3 Effect of the MAPK inhibitor PD98059 on leptin secretion

(A) Primary adipocytes were pre-treated with 50 μM PD98059 for 30 min followed by 2 h treatment with 1 μg/ml insulin. Results are means ± S.E.M.; n = 10. **P < 0.01. (B) Representative immunoblots showing the effect of 50 μM PD98059 on MAPK phosphorylation in primary adipocytes. p-MAPK, phospho-MAPK.

Chemicals

Antibodies against the following were used: calnexin and the KDEL motif (Abcam); synataxin 6 s (Becton Dickinson); golgin 97 (Invitrogen); and caveolin 1, phospho-Akt (Ser173), Akt, phospho-MAPK and MAPK (Cell Signaling Technology). Insulin, wortmannin, Akti, ionomycin and PD98059 were purchased from Merck Millipore. EGTA/AM, BAPTA/AM and collagenase type IV were obtained from Invitrogen. Proteinase inhibitor cocktail was from Roche. All other chemicals, unless otherwise mentioned, were obtained from Sigma–Aldrich.

Subcellular fractionation

Subcellular fractionation was performed as described in [4] with modifications. Briefly, primary adipocytes were isolated from 20 male adult mice and pooled together. After being washed once with 20 ml TES (Tris/EDTA/sucrose) buffer (20 mM Tris/HCl, 1 mM EDTA and 8.7% sucrose; pH 7.4) and suspended in TES buffer, the adipocytes were homogenized in a pre-cooled (4°C) Potter–Elvehjem grinder (Corning) with 20 hand strokes and centrifuged at 10000 g for 1 min at room temperature, and an additional 15 min at 4°C. The solidified fat at the top was removed.
and discarded. The supernatant was centrifuged at 27600 g for 30 min at 4 °C. The pellet contained the HDM and was suspended to be centrifuged again at 27600 g. The resulting supernatant was further centrifuged for 75 min at 200000 g (4 °C) to pellet the LDM. The pellet was suspended and centrifuged again at 200000 g. The pellet of the first centrifugation (10000 g) was suspended in TES buffer (2 ml in a Potter–Elvehjem grinder; 20 strokes), loaded on to the sucrose cushion (38.5 %) and centrifuged for 60 min at 100000 g (4 °C) in a swing-out rotor. The resulting pellet contained the nuclei and mitochondria. The PM was collected from the top of the sucrose cushion, suspended in TES buffer and pelleted by centrifugation at 31000 g for 60 min. All the pellets were suspended and homogenized in 0.4–1 ml TES buffer. For differentiated 3T3-LV adipocytes, cells were washed at 8000 g for 5 min. The cells were centrifuged in a Potter–Elvehjem grinder with 15 hand strokes. Homogenized cells were centrifuged at 8000 g for 15 min. The subsequent centrifugation steps were identical to those for the primary adipocytes.

Trypsin protection assay

Trypsin protection assays were performed as described previously [5] with slight modifications. Briefly, 3T3-L1 adipocytes were rinsed three times with ice-cold PBS and scraped with a rubber policeman, harvested in ice-cold PBS and collected by centrifugation at 800 g at 4 °C for 5 min. The cells were resuspended in 500 μl of ice-cold buffer (2 mM MgCl₂, 2 mM CaCl₂, 50 mM NaCl, 250 mM sucrose and 50 mM Tris/HCl, pH 7.4) and homogenized using 20 strokes of a 25-gauge needles. Cell lysate was collected by centrifugation at 10000 g at 4 °C for 10 min. The samples were treated for 2 h at 4 °C with or without 0.5 mg/ml trypsin or Triton X-100 (1 % w/v). Samples were separated by SDSPAGE (10 % gel) and analysed by Western blotting using the indicated antibodies.

Immunocytochemistry

3T3-LV cells were cultured and differentiated to days 7–9, plated on to poly-L-lysine-coated coverslips and cultured overnight. The cells were washed with PBS, fixed in PBS containing 4 % paraformaldehyde for 10 min, permeabilized in PBS containing 0.2 % saponin for 5 min and blocked in blocking buffer (3 % BSA and 3 % goat serum in PBS) for 1 h at room temperature. The cells were then probed by the antibodies indicated and fluorescence-conjugated secondary antibodies (Invitrogen). The coverslips were mounted on to slides and observed by confocal microscopy (Nikon).

REFERENCES