Ph.D. THESIS

INVESTIGATION OF PATHOPHYSIOLOGICAL FACTORS AND PHARMACOLOGICAL TARGETS FOR AMELIORATING LEPTIN RESISTANCE IN OBESITY

MINA THON

DEPARTMENT OF PHARMACOTHERAPY

GRADUATE SCHOOL OF BIOMEDICAL AND HEALTH SCIENCES

HIROSHIMA UNIVERSITY
2016

Contents

Abstract

A lack of physical activities and excess in food intake in modern life style play important role in the development of overweight and obesity. Yet the underlying mechanisms are still being elucidated in details. Leptin, an adipocyte-derived hormone, centrally regulates energy homeostasis by accelerating energy expenditure and suppressing food intake. However, leptin resistance is a hallmark of obese subjects. We and others have previously reported the involvement of endoplasmic reticulum (ER) stress in the pathophysiology of leptin resistance. Thus, being able to identify the factors involved in and the novel target for ameliorating leptin resistance would provide a potential therapeutic approach for obesity and related diseases. The aims of the present studies are to identify physiological factors and pharmacological targets for ameliorating leptin resistance in obesity.

First, we identified pathophysiological factors mediated leptin resistance including excess in dehydroascorbic acid (DHAA) and excess in saturated fatty acids (SFAs) in defective leptin signaling. DHAA, an oxidized form of vitamin C, was found to be diabetogenic. In the current study, we hypothesized the drawback effect of DHAA on defective leptin signaling. A human neuroblastoma cell line stably transfected with the Ob-Rb leptin receptor (SH-SY5Y-ObRb), was treated with DHAA and leptin signaling was then analyzed. Interestingly, we found that an elevated of DHAA inhibited leptin-induced STAT3 phosphorylation. We found that DHAA increased mRNA expression levels of ER stress-related genes such as glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP) and X-box-binding protein 1 (XBP1) splicing. Theses results suggested that DHAA plays important role in the development of leptin resistance through ER stress.

High-fat rich diet is associated with lipotoxicity and ER stress. Accordingly, using SH-SY5Y-ObRb cells, we found that the principle source of SFAs, palmitate inhibited leptin-induced STAT3 in neuronal cells. To further confirm the harmful effect of excess SFAs on leptin signaling, an inhibition of stearoyl-CoA desaturase-1 (SCD1), an enzyme that catalyzes the synthesis of monounsaturated fatty acids (MUFAs) from SFAs, was then evaluated. To achieve this hypothesis, disruption of SCD1 was carried out through the use of SCD1 inhibitor, CAY10566 and SCD1 knockdown, respectively. As expected, leptin-induced phosphorylation of STAT3 was inhibited in SCD1 inhibitor-treated and SCD1-knockdown SH-SY5Y-ObRb cells. Moreover, SCD1 inhibitor induced ER stress responsive gene, GRP78 in HEK293-ObRb cells. Thus, excess in SFA levels is involved in the pathophysiology of leptin resistance.

Second, we aimed to search for pharmacological targets for ameliorating leptin resistance in obesity. ER stress is known to implicate in the pathogenesis of multiple diseases including leptin resistance. Therefore, an intervention that alleviates ER stress would provide a useful therapeutic approach for obesity and ER stress-related diseases. In response to ER stress, cells activate an adaptive response termed unfolded protein response (UPR). One of the protective mechanisms of UPR is the induction of ER chaperone, i.e., glucose-regulated protein 78 (GRP78). GRP78, a major chaperone located in the lumen of the ER, protects against ER stress by promoting folding of proteins to prevent aggregation. Therefore, the induction of GRP78 is critical in maintaining normal cell functions. In the present study, using SH-SY5Y-ObRb cells we demonstrated that leptin induces GRP78 expression. In addition, leptin-induced GRP78 was not depended on its classical upstream activation pathway, IRE1-XBP1, as we could not detect IRE1 and XBP1 activation in our

experimental conditions. In contrast, we showed that PI3K, LY294002, and mTOR inhibitor, rapamycin, blocked leptin's effect on the induction of GRP78. Of note, ER-apoptotic marker, CHOP, was not induced by leptin treatment in our experiments. Thus, leptin may specifically induce the expression of GRP78. Moreover, we showed that leptin protects against ER stress-induced cell death in human neuronal cell line. Therefore, leptin may induce the expression of GRP78, thereby protecting against ER stress related to obesity.

To date, the overlaps in the regulation of glucose and energy homeostasis have been reported between leptin and insulin. However, the effects of insulin on leptin's actions in hypothalamus have not yet been completely elucidated. In the current work, we found that insulin potentiates leptin-induced STAT3 phosphorylation through GRP78. The role of GRP78 in leptin's actions was also confirmed by defective leptin-induced STAT3 phosphorylation in SH-SY5Y-ObRb and HEK293-ObRb cells in which GRP78 was knocked down. Indeed, we found that the overexpression of GRP78 enhanced leptin-induced STAT3 phosphorylation. Based on the important role of GRP78 on leptin-induced signal, we also investigate the detailed mechanisms of which GRP78 modulated leptin signaling. We hypothesized that GRP78 may directly bind to ObRb long isoform of leptin receptor which leads to potentiation of leptin-induced signals. To do this, we first co-overexpressed ObRb receptor and GRP78 in HEK293T cells prior to an immunoprecipitation of ObRb. GRP78 was then analyzed by western blotting. As the result, we found that GRP78 binds to the long isoform of leptin receptor ObRb.

Our results highlight the novel pathophysiological factors of leptin resistance such as excess in SFAs and DHAA. ER stress, indeed, may play important role in the pathophysiology of leptin resistance induced by DHAA or SFAs. Moreover, we

demonstrated that leptin upregulate the expression of GRP78 through the PI3K-mTOR pathway, and protects against ER stress. We also showed that GRP78 plays an important role in leptin's actions. We demonstrated that insulin enhanced the leptin-induced activation of STAT3 by inducing GRP78. To this, GRP78 would be a new mediator between leptin and insulin in the regulation of energy homeostasis. Together our data may provide useful information for further understanding of the pathophysiological factors as well as target molecule for attenuating leptin resistance in obesity.

Background

Over the last few decades, the incidence of obesity has been increasing at a rapid rate (Kelly *et al*., 2005). Obesity is a risk factor for life-threatening diseases such as diabetes mellitus, hypertension, dyslipidemia, nonalcoholic fatty liver disease, cardiovascular disease, and other chronic diseases (Malnick *et al*., 2006). The hypothalamus plays important roles in the regulation of energy balance (Morton *et al*., 2006), which is crucial for combating weight gain (Hall *et al*., 2012).

Leptin, a hormone secreted by white adipocytes, is considered to have anti-obesity effects by suppressing food intake and enhancing energy expenditure (Ahima *et al*., 1996; Halaas *et al*., 1995). In the hypothalamus, the binding of leptin to the long isoform of its receptor, ObRb, leads to an activation of multiple signaling cascades, such as Janus kinase 2-signal transducer and activator of transcription 3 (JAK2-STAT3) (Fei *et al*., 1997), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) (Takahashi *et al*., 1997; Cui *et al*., 2006), and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathways (Niswender *et al*., 2001). The major anti-obesity effects of leptin are initiated by the phosphorylation of JAK2-STAT3 pathway. Although leptin has been considered a potential anti-obesity candidate protein, the majority of people with obesity are unresponsive to leptin's action, i.e., leptin resistant (Münzberg *et al*., 2005). However, the underlying mechanisms are still being elucidated. Currently, studies on the underlying mechanism of leptin resistance have become a central focus in the search for a possible treatment of obesity.

Endoplasmic reticulum (ER) is an important organelle for assembling, folding, maturation, and transportation of proteins (Helenius *et al*., 1992). Disruption of these processes, due to various factors that induce an accumulation of unfolded or

misfolded proteins, leads to ER stress. In response to ER stress, an adaptive response, named unfolded protein response (UPR), will be activated to restore normal function of the cells by attenuating protein translation to reduce the loading of unfolded proteins in the ER through the promotion of protein folding and activation of degradation pathways (Harding *et al*., 1999; Mori, 2000; Friedlander *et al*., 2000). However, prolonged and severe ER stress activates apoptotic signals and causes cell death (Wang *et* al., 1996; Zinszner *et al*., 1998; Nakagawa *et al*., 2000). The UPR system signals mediate by activating ER transmembrane sensors, such as the inositol-requiring protein-1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK), and activating transcription factor-6 (ATF6) arms (Ron *et al*., 2007). Of note, Glucose-regulated protein 78 (GRP78), a major up-regulated UPR chaperone located in the lumen of the ER, is a required protein to maintain ER capacity and protect against ER stress by assisting in protein folding, which prevents aggregation (Kaufman, 1999; Rao *et al*., 2002; Lee, 2005).

ER stress is known to implicate in the pathogenesis of a wide range of diseases (Sai *et al*., 2002; Imai *al*., 2001; Harding *et al*., 2002). Our group (Hosoi *et al*., 2008), and others (Ozcan *et al*., 2009; Won *et al*., 2009), reported the involvement of ER stress in the pathophysiology of resistance in obesity.

Taken together, an identification of the mechanisms of obesity pathogenesis as well as the molecules that control food intake would be useful for the therapeutic approach of obesity. Therefore, the aims of our studies are to investigate the pathophysiological factors and the target molecule for attenuating of leptin resistance associated with obesity.

Part 1: Dehydroascorbic acid (DHAA)

1.1. Introduction

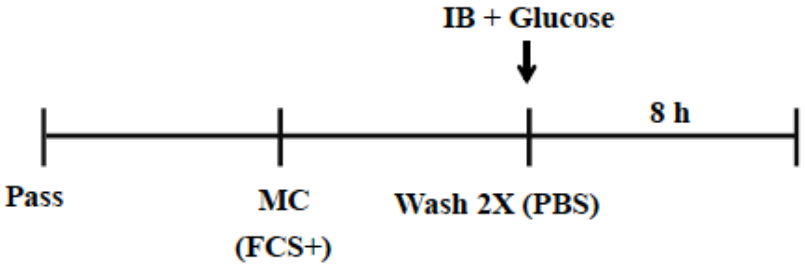
Vitamin C, a water-soluble vitamin, exists in two major forms: the reduced form, ascorbic acid (AA), and the oxidized form, dehydroascorbic acid (DHAA). AA is transported across the cell membrane via a family of sodium/vitamin C co-transporters (SVCT). AA is oxidized to DHAA outside the cells, and then enters the cells through the glucose transporter (GLUT) (Vera *et al.,* 1993; Ngkeekwong *et al*., 1997). The reduction of DHAA to AA inside the cells is necessary for maintaining sufficient intracellular levels of AA, which is essential for its antioxidant properties (Bendich *et al*., 1986; Frei *et al*., 1989). High plasma levels of DHAA are found in patients with diabetes (Sinclair *et al*., 1999; Som *et al*., 1981). In addition, DHAA was also suggested to be diabetogenic (Patterson, 1949). Currently, little is known about the mechanisms of the harmful effects of DHAA. In the present study, we hypothesized the possibility that high levels of DHAA might cause defective anti-obesity action of leptin through ER stress; thereby promote leptin resistance in human neuronal cells.

1.2. Materials and methods

1.2.1. Reagents

Human leptin was obtained from Enzo Life Science (NY). (L)-Dehydroascorbic acid was purchased from Sigma-Aldrich (St. Louis, MO).

1.2.2. Stimulation of dehydroascorbic acid (DHAA)



DHAA was prepared in incubation buffer (IB), 15 mM HEPES, 135 mM NaCl, 5 mM KCL, 1.8 mM CaCl2, 0.8 mM MgCl2, pH 7.4 (S. KC *et al*, 2005). Cells were washed twice with PBS and changed to IB in the presence of glucose (0.1 mM) followed by DHAA stimulation (1mM) for 8 h.

1.2.3. Cell culture

Human neuroblastoma (SH-SY5Y) cell line stably transfected with the Ob-Rb long form of the leptin receptor (SH-SY5Y-ObRb) (Hosoi, T. *et al,* 2006) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37°C in humidified 5% CO_2/95% air.

1.2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from fresh cells using TriPure Isolation Reagent (Roche). cDNA was reverse transcribed from 2 µg of total RNA by using 25 U of Supercript Reverse Transcriptase (ReverTra Ace) and 0.25 µg of Oligo(dt) 12-18 primer (Life Technology) in a reaction mixture containing buffer, 1 mM dNTP mix, 10 mM DTT and 40 U of RNase inhibitor. The total RNA and Oligo(dt) 12-18 primer were incubated at 70 ^{o}C for 10 min before RT. After incubation for 1.5 h at 46 ^{o}C, the RT reaction was terminated by denaturing the reverse transcriptase for 5 min at 100 ^{o}C. Regarding PCR amplification, 1.2 µl of cDNA was added to 10.8 µl of a

reaction mix containging 0.2 μM of each primer, 0.2 μM of dNTP mix, 0.6 U of Taq polymerase and reaction buffer. PCR was performed in a DNA Thermal Cycler (MJ Research, PTC-220 and TaKaRa PCR Thermal Cycler Dice TP 600). The following primers were used: XBP-1; upstream, 5'- CCT TGT AGT TGA GAA CCA GG -3', and downstream, 5'- GGG GCT TGG TAT ATA TGT GG -3'. CHOP; upstream, 5'-GCA CCT CCC AGA GCC CTC ACT CTC C-3', and downstream, 5'-GTC TAC TCC AAG CCT TCC CCC TGC G-3'. GRP78; upstream, 5'-TGC TTG ATG TAT GTC CCC TTA-3', and downstream, 5'-CCT TGT CTT CAG CTG TCA CT-3'. GAPDH; upstream, 5'-AAA CCC ATC ACC ATC TTC CAG-3', and downstream 5'-AGG GGC CAT CCA CAG TCT TCT-3' was used as an internal control.

The PCR products (10 μl) were resolved by electrophoresis in an 8% polyacrylamide gel in TBE buffer. The gel was stained with ethidium bromide and photographed under ultraviolet light.

1.2.5. Western Blotting Analysis

Western blotting was performed as described previously (Hosoi *et al*., 2006). Cells were washed with ice-cold phosphate-buffered saline and lysed in buffer containing 10 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40 for 20 min. Lysates were centrifuged at 15,000 rpm at 4°C for 20 min, and supernatants were collected. Samples were boiled with Laemmli buffer for 3 min, fractionated using SDS-polyacrylamide gel electrophoresis, and transferred at 4°C to nitrocellulose membranes. Five-percent skimmed milk was prepared in 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20 (TBST) buffer, and was then used to block

membranes at room temperature for 1 h. The membranes were then incubated with rabbit anti-phospho-STAT3 (Tyr705) (#9145S, Cell Signaling) and rabbit anti-STAT3 (#9132, Cell Signaling), antibodies, and this was followed by incubation with an anti-horseradish peroxidase-linked antibody. Peroxidase was detected using enhanced chemiluminescence with an ECL system (Amersham).

phospho-STAT3 (Tyr705) (#9145, Cell Signaling)

- Blocking: 5 % milk (TBST); 1h, room temperature (RT)
- 1st antibody: diluted 1/2,000 in 5 % BSA; overnight
- Secondary antibody: anti-rabbit, diluted 1/5,000 in 5% milk (TBST); 1 h

STAT3 (# 9132, Cell Signaling)

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/1000 in TBST; overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

1.2.6. Statistics

A student's *t*-test was used for comparison between two groups. Significance was defined as a *P* value.

1.3. Results

1.3.1. Dehydroascorbic acid induced leptin resistance

DHAA is transported into the cells through GLUT (Rumsey *et al*., 1997). It was known that DHAA competes with glucose for an uptake (Siushansian *et al*., 1997). Based on these concepts, we speculated that DHAA would compete with glucose to be transported into the cells. To achieve this, we used 0.1 mM glucose and 1 mM DHAA in our experiments. SH-SY5Y-ObRb cells were washed twice with PBS,

changed to incubation buffer (IB), and stimulated with DHAA (1 mM, 8 h), following leptin stimulation (0.03 μg/ml, 15 min). The result showed that leptin-induced STAT3 phosphorylation was significantly inhibited by DHAA in SH-SY5Y-ObRb cells (Fig. 1).

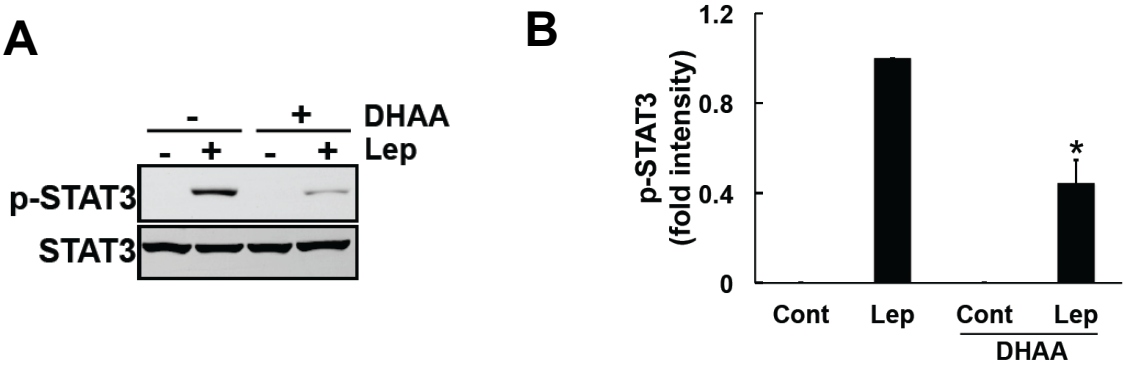


Figure 1. DHAA induced leptin resistance in neuronal cells.

SH-SY5Y-ObRb cells were washed with PBS twice and changed to incubation buffer (IB) in the presence of glucose (0.1 mM). Cells were stimulated with DHAA (1 mM) for 8 h, followed by leptin (0.03 μg/ml, 15 min). Phospho-STAT3 (Tyr705) and STAT3 levels were analyzed by western blotting. **A.** DHAA inhibited leptin-induced STAT3 phosphorylation. **B.** A densitometric analysis of phospho-STAT3 (Tyr705) was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n = 4). * $P < 0.05$.

1.3.2. Dehydroascorbic acid induced ER stress

We and others have previously reported that ER stress is involved in the pathophysiology of leptin resistance (Hosoi *et al*., 2008; Ozcan *et al*., 2009; Won *et al*., 2009). Since we found that DHAA caused leptin resistance, we further examined whether DHAA induces ER stress. The activation of UPR results in an upregulation of UPR-related genes, such as GRP78, an ER chaperone, or the splicing of X-box-binding protein 1 (XBP1). We therefore analyzed ER stress-responsive genes. SH-

SY5Y-ObRb cells were washed twice with PBS, changed to incubation buffer (IB), and stimulated with DHAA (1 mM) in the presence of glucose 0.1 mM for 8 h. The expression of UPR-related genes was then analyzed by RT-PCR. We showed that DHAA induced GRP78 and XBP1 splicing, signifying activation of ER stress (Fig. 2).

In the case of severe ER stress, or UPR malfunction, C/EBP homologous protein (CHOP), an ER stress-induced apoptotic transcription factor, is activated (Zinszner *et al*., 1998; Maytin *et al*., 2001). As we found that DHAA activated ER stress-related genes, such as GRP78 and spliced-XBP1 mRNA levels, we next investigated whether DHAA also induced CHOP. DHAA (1 mM, 8 h) significantly induced CHOP mRNA expression levels in SH-SY5Y-ObRb cells (Fig. 2). These results suggested that DHAA may induce ER stress in neuronal cells.

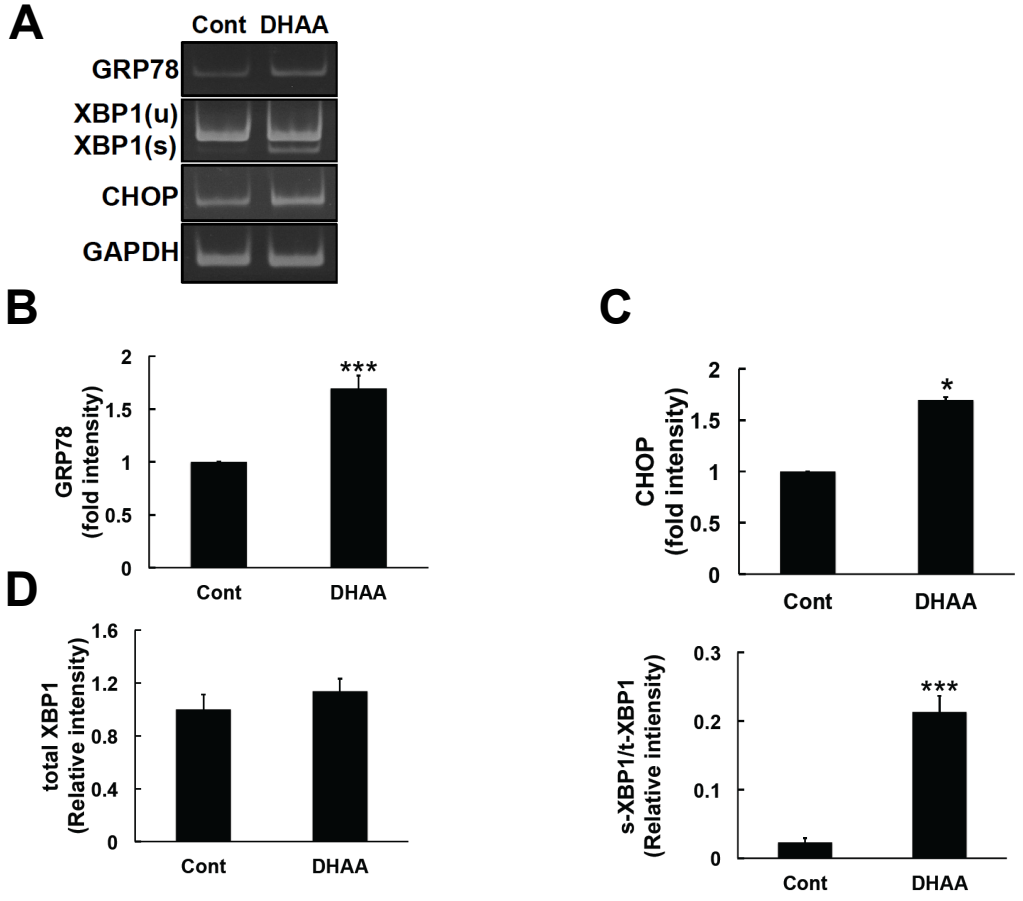


Figure 2. DHAA activated UPR-related genes in neuronal cells.

A. SH-SY5Y-ObRb cells were washed with PBS twice and changed to incubation buffer (IB) in the presence of glucose (0.1 mM). Cells were stimulated with DHAA (1 mM) for 8 h, and the expression level of GRP78, CHOP and spliced XBP1 were analyzed by RT-PCR. DHAA induced GRP78, CHOP and a spliced form of XBP1 mRNA expression **B**. A densitometric analysis of GRP78 mRNA was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n = 4). *** $P < 0.001$. **C.** A densitometric analysis of CHOP mRNA was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n = 4). * $P < 0.05$. **D.** A densitometric analysis of spliced/total XBP1 and total XBP1 mRNA (unspliced and spliced XBP1 mRNA) was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n = 4). *** $P < 0.001$.

1.4. Discussion

We found that increased DHAA, an oxidized form of vitamin C, causes ER stress and induces leptin resistance in a human neuroblastoma cell model. As a result of the conversion of DHAA to AA inside the cell (Bendich *et al*., 1986), the intracellular concentration of reducing agents such as NADPH and glutathione (GSH) is decreased, and oxidative stress will be generated (Siushansian *et al*., 1997; May *et al*., 2001). Oxidative stress is linked to neuronal cell death (Ratan *et al*., 1995), apoptosis (Zamzami *et al*., 1995) and ER stress (Holtz *et al*., 2006), one of the core events in the pathogenesis of leptin resistance in obesity (Hosoi *et al*., 2008; Ozcan *et al*., 2009; Won *et al*., 2009). Of note, neurons are sensitive to DHAA-induced cell death (García-Krauss *et al*., 2015). In the present study, we found that DHAA upregulated

the expression of ER stress-responsive genes such as GRP78, CHOP, and spliced XBP1. This effect of DHAA may be related to the pathophysiology of leptin resistance, as signified by the inhibition of leptin-induced STAT3 phosphorylation in neuronal cells by DHAA.

Part 2: Saturated fatty acids (SFAs)

2.1. Introduction

Elevated plasma concentration of free fatty acids (FFAs) are observed in obesity and type 2 diabetes (Roden *et* al., 1996; Eizirik *et al*., 2008). Current evidence suggests that excess in food intake may contribute to the development of ER stress and activation of the UPR signaling pathway (Ozcan *et al.*, 2004; Ozcan *et al*., 2009). High-fat rich diets drove the link between lipotoxicity and ER stress. However, the underlying mechanism of how excess FFA contributes to leptin resistance remains poorly understand. In the present study, we hypothesized the possibility that elevated FFA might interfere with anti-obesity action of leptin in hypothalamus, which in turn might provoke leptin resistance

2.2. Materials and methods

2.2.1. Reagents

Human leptin was obtained from Enzo Life Science (NY). Palmitate was purchased from Tokyo Chemical Industry. BSA was provided by Sigma (MO). SCD1 inhibitor (CAY10566) was obtained from Cayman Chemical.

2.2.2. Palmitate preparation

Palmitate was prepared as described previously (Mayer et al., 2010). Briefly, 100 mM solution of palmitate was prepared by dissolving 27.8 mg sodium palmitate in 1 ml sterile water by alternating heating and vortexing until the palmitate was dissolved, once the solution reached 70^0C. Immediately, 200 µl of the 100-mM palmitate solution were added to 3.8 ml of serum-free medium containing 0.1 % NEFA-free

BSA to get a final concentration of 5 mM palmitate solution. The 5 mM palmitate solution was shaken at 140 rpm at 40^0C for 1 h. The palmitate solution was then immediately used to treat the cells. Serum-free medium containging 0.1 % BSA was used as the control.

2.2.3. Cell culture

Human neuroblastoma (SH-SY5Y) and human HEK293 cell lines stably transfected with the Ob-Rb long form of the leptin receptor (SH-SY5Y-ObRb and HEK293-ObRb, respectively) (Hosoi, T. *et al,* 2006) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37°C in humidified 5% CO_2/95% air.

2.2.4. Western Blotting Analysis

As described in 1.2.5. Additional antibodies used:

anti-SCD1 (#2438, Cell Signaling)

- Blocking: 3 % milk (TBST); 1h, RT
- 1^{st} antibody: diluted 1/1,000 in 5 % BSA; two overnight
- Secondary antibody: anti-rabbit, diluted 1/5,000 in 5% milk (TBST); 1 h

anti-GAPDH (ACR001P, Acris).

- Blocking: 5 % milk (TBST); RT
- 1^{st} antibody: diluted 1/2, 000 in TBST; overnight
- Secondary antibody: anti-mouse, diluted 1/5, 000 in 5% milk (TBST); 1 h

2.2.5. Statistics

As described in 1.2.6.

2.2. Results

2.2.1. Palmitate induced leptin resistance

The SFAs i.e., palmitate has been shown to induce leptin resistance (Kleinridders *et al*., 2009; Zheng *et al*., 2013). Using SH-SY5Y-ObRb cell line, we showed that palmitate (100 μM, 2 h) inhibited leptin-induced phosphorylation of STAT3 (Fig. 3).

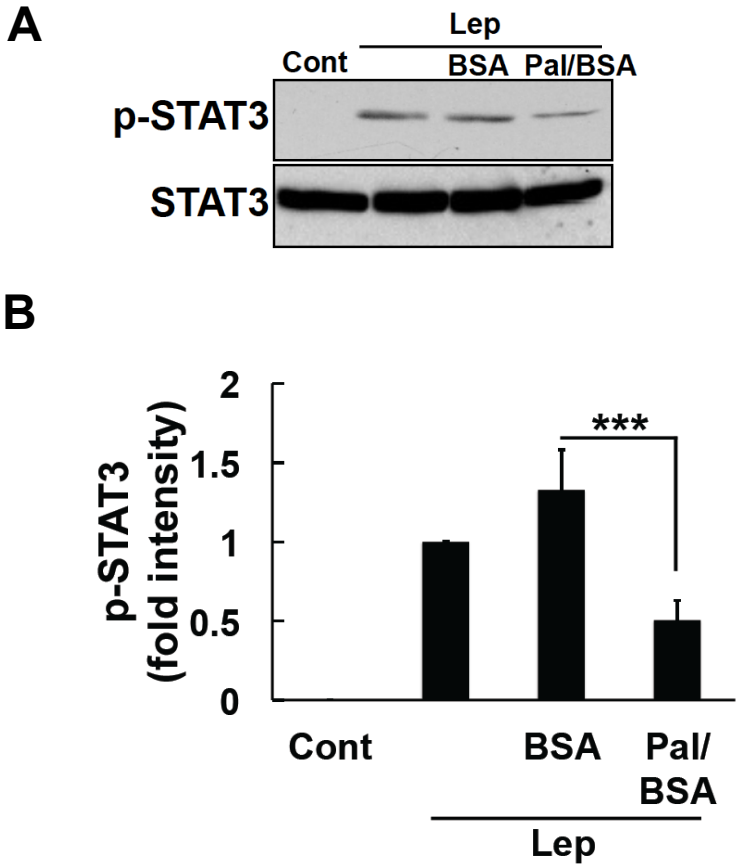


Figure 3. Palmitate attenuated leptin signaling in neuronal cells.

A. SH-SY5Y-ObRb cells were treated with 0.1% BSA vehicle or final concentration of 100 μM palmitate for 2 h followed by leptin 0.03 μg/ml for 15 min. Phospho-STAT3 (Tyr705) and STAT3 levels were analyzed by western blotting. **B.** A densitometric analysis of phospho-STAT3 (Tyr705) was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n = 4). *** $P < 0.001$.

2.2.2. Stearoyl-CoA desaturase-1 (SCD1) inhibitor induced leptin resistance

SCD1 catalyzes the synthesis of monounsaturated fatty acids from saturated fatty acids. Therefore, we reasoned that inhibition of SCD1 would increase SFAs levels. We further assessed the function of SCD1 on leptin signaling in neuronal cells. To do this, we used SCD1 inhibitor CAY10566 (CAY). SH-SY5Y-ObRb cells were treated with CAY (10 μM, 2, 4 and 8 h), followed by leptin (0.03 μg/ml, 15 min). We then analyzed leptin-induced signal by western blotting. We found that SCD1 inhibitor inhibited leptin-induced STAT3 phosphorylation in time-dependent manner (Fig. 4).

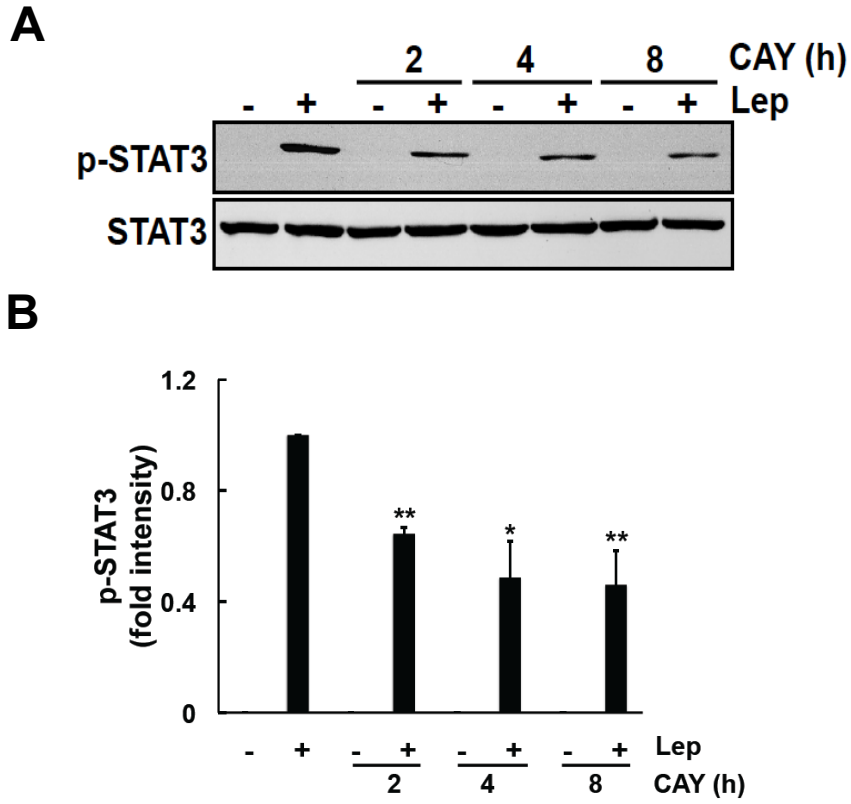


Figure 4. SCD1 inhibitor attenuated leptin signaling in neuronal cells.

A. SH-SY5Y-ObRb cells were treated with CAY10566 (CAY) 10 μM for 2, 4 and 8 h. Leptin 0.03 μg/ml was stimulated for another 15 min. Phospho-STAT3 (Tyr705) and STAT3 levels were analyzed by western blotting. **B.** A densitometric analysis of phospho-STAT3 (Tyr705) was performed using image analysis software. Data are

expressed as the mean ± S.E. of 4 independent experiments (n = 4). * $P < 0.05$; ** $P < 0.01$.

2.2.3. SCD1 knockdown induced leptin resistance

To further confirm the involvement of SCD1 in leptin resistance, we performed SCD1 knockdown experiment in SH-SY5Y-ObRb cells. Cells were transfected with SCD1 siRNA (5 nM) for 72 h. As an indicator of transfection efficacy, the expression of SCD1 was subjected to a western blotting analysis. We confirmed that SCD1-specific siRNA efficiently inhibited its expression in SH-SY5Y-ObRb cells (Fig. 5). Under these conditions, we analyzed leptin-induced STAT3 signaling. We found that the knockdown of SCD1 significantly reduced the leptin-induced phosphorylation of STAT3 in the SH-SY5Y-ObRb cells (Fig. 5). Together, theses results indicated that excess in SFAs plays important role in pathophysiology of leptin resistance.

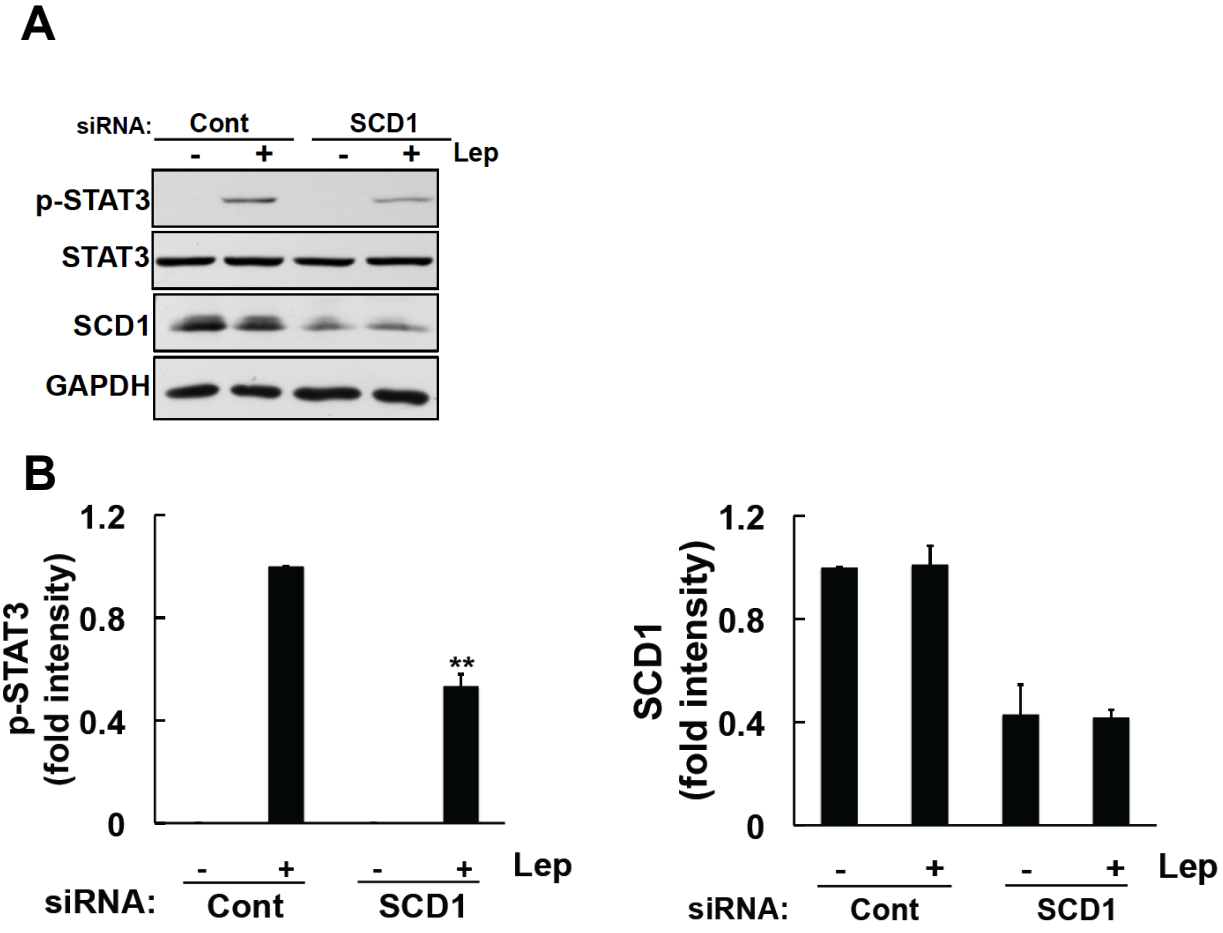


Figure 5. Leptin signaling was attenuated in SCD1-knocked down neuronal cells.

A. SH-SY5Y-ObRb cells were transfected with control or SCD1 siRNAs (5 nM) for 72 h. Seventy-two hours after the transfection, cells were treated with leptin (0.03 μg/ml, 15 min). Phospho-STAT3 (Tyr705), STAT3, SCD1 and GAPDH levels were analyzed by western blotting. Leptin-induced STAT3 phosphorylation was attenuated in SCD1-knocked down cells. **B.** A densitometric analysis of phospho-STAT3 (Tyr705) and SCD1 was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n=4). ** $P < 0.01$.

2.2.4. SCD1 inhibitor induced ER stress

Because we found that SCD1 inhibitor caused leptin resistance, we investigated whether SCD1 inhibitor induces ER stress. We therefore analyzed an ER stress-response gene, GRP78. HEK293-ObRb cells treated with CAY (10 μM) for 2, 4 and 8 h. CAY up-regulated the expression of GRP78 (Fig. 6). This result suggested that inhibition of SCD1 may induce ER stress.

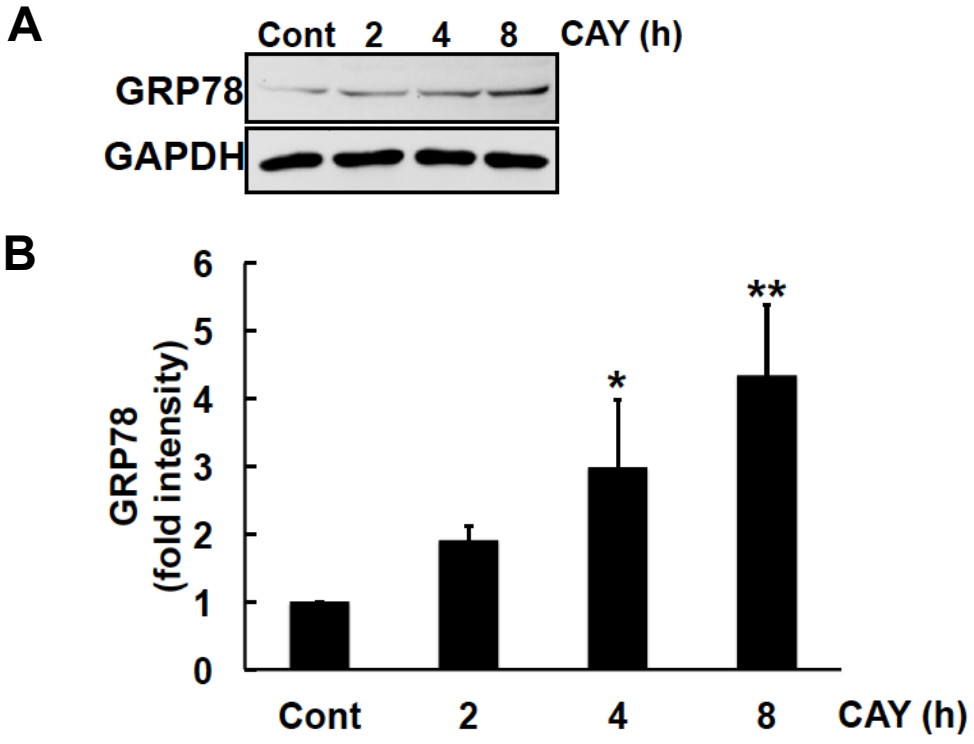


Figure 6. SCD1 inhibitor induced ER stress in HEK293-ObRb cells.

A. HEK293-ObRb cells were treated with SCD1 inhibitor, CAY10566 (CAY) 10 μM for 2, 4 and 8 h. GRP78 and GAPDH levels were analyzed by western blotting. **B.** A densitometric analysis of GRP78 was performed using image analysis software. Data are expressed as the mean ± S.E. of 3 independent experiments (n = 3). * $P < 0.05$, ** $P < 0.01$.

2.4. Discussion

Current evidence suggests that a high-saturated-fat diet induces leptin resistances (Lin *et al*., 2000) and contribute to the development of ER stress and activation of the UPR signaling pathway (Zhang *et al*., 2008). Palmitate, a principal source of saturated fatty acids in diet, is known to have a strong lipotoxic effects in induction of inflammation and ER stress (Weigert *et al*., 2004; Borradaile *et al*., 2006). SCD1, on the other hand, plays a key role in modulating the toxicity effects of SFAs (Pinnamaneni *et al*., 2010; Peter *et al*., 2008). It was previously reported that palmitate induced ER stress (Karaskov *et* al., 2006; Lu *et* al., 2012; Yin *et al., 2015*) and leptin resistance (Kleinridders *et al*., 2009; Zheng *et al*., 2013). ER stress is one of the core mechanisms of the pathogenesis of leptin resistance in obesity (Hosoi et al., 2008; Ozcan et al., 2009; Won et al., 2009). In the present study, we figured out the hypothesis of which the loss of SCD1 activity impaired leptin-induced signal, as signified by the inhibition of leptin-induced STAT3 in response to SCD1 inhibitor and knocking down of SCD1. These results suggested that SCD1 may play important role in the regulation of saturation levels thereby prevent lipotoxicity. In this aspect, elevated SCD1 provided a protective effect against fatty acid-induced insulin resistance (Pinnamaneni *et al*., 2006). Moreover, SCD1 knockout mice displays and inflammation and ER stress (Flowers *et al*., 2008). Accordingly, we showed in the

present study that SCD1 inhibitor induces ER stress, as signified by the induction of GRP78 (Fig. 6). Thus, ER stress may account for the impairment of leptin signaling induced by reduction of SCD1. Of note, overexpression of SCD1 in rat muscle cells protected against fatty acid-induced insulin resistance (Pinnamaneni *et al*., 2006). From MIN6 β-cell line, upregulation of SCD1 expression results in resistance to SFAs-induced cell death (Busch *et al*., 2005). So, the inhibition of conversion of SFAs to MUFAs by SCD1 knockdown may cause harmful effect in our studies. However, controversies on the role of SCD1 have been reported. SCD1 expression levels are strongly upregulated in obese mice (Asilmaz *et al*., 2004). The inhibition of SCD1 activity in mice, as signified by the use of SCD1-specific antisense oligonucleotide inhibitors (ASOs) and deletion of SCD1 (SCD1-/-), prevented diet-induced obesity (Jiang *et al.,* 2005, Ntambi *et* al., 2002). Since SCD1 is thought to mediate fat storage in adipose tissue, it has been suggested that attenuation of SCD1 activity might inhibit body weight through the blockage of fat storage (Popeijus *et al*., 2008). The controversial biological outcome of SCD1 may be explained by difference in specific organ's SCD1 activity. Notably, we used human neuronal cellular model in the present study. Therefore, further studies should be conducted in brain-specific deletion of SCD1 models for more understanding about the function of SCD1 in the CNS.

Part 3: Leptin induced GRP78 expression through the PI3K-mTOR pathway in neuronal cells

3.1. Introduction

ER stress is one of the core mechanisms mediated the pathophysiology of leptin resistance, a pathogenesis of obesity (Hosoi *et al*., 2008; Ozcan *et al*., 2009; Won *et al*., 2009). Still, the mechanisms underlying ER stress and activation of UPR signaling in obesity have yet to be fully illustrated. In the present study, we hypothesized that leptin may be able to activate the UPR in neuronal cells.

3.2. Materials and methods

3.2.1. Reagents

Rapamycin was purchased from Santa Cruz (CA). Tunicamycin (Tm) and thapsigargin (Tg) were obtained from Wako Pure Chemical Ltd. (Japan). LY294002 was purchased from Sigma (MO). PD98059 was provided by Research Biochemical International (MA).

3.2.2. Cell culture

As described in 1.2.3.

3.2.3. Lactate Dehydrogenase Leakage (LDH) Assay

The viability of cells was estimated by the lactate dehydrogenase (LDH) leakage method using a cytotoxicity detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's directions. LDH activity was measured at the optimal density 492 nm.

3.2.4. WST-1 Assay

Cell viability was measured using WST-1 reagent (Roche) according to the manufacturer's directions, a mixture of 100 µl medium and 10 µl cell proliferation WST-1 reagent was added in to the cells, followed by an incubation at 37°C and 5% CO_2 for 4 h. The samples were then analyzed for optical density (440 - 600 nm).

3.2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

As described in 1.2.4.

3.2.6. Western blotting analysis

As described in 1.2.5. Additional antibodies used:

anti-phospho-Akt (Thr308) (#9275, Cell Signaling),

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/1, 000 in 5 % BSA (TBST); two overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

rabbit anti-phospho-ERK (Thr202/Tyr204) (39191S, Cell Signaling)

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/1, 000 in 5 % BSA (TBST); overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

rabbit anti-CHOP (G1708, Santa Cruz),

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/500 in TBST; two overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

rabbit anti-phospho-IRE1α (phospho S724) (ab48187, Abcam),

- Blocking: 0.5 % BSA (TBST); 1h, RT

- 1st antibody: diluted 1/1, 000 in TBST; two overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 1 % BSA (TBST); 1 h

anti-KDEL (Enzo Life Sciences; #02231116),

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/1, 000 in TBST; overnight
- Secondary antibody: anti-mouse, diluted 1/5, 000 in 5% milk (TBST); 1 h

anti-phospho-S6K (Thr389) (#9234, Cell Signaling),

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/1, 000 in 5 % BSA (TBST); overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

anti-ERK1/2 (SPC-120C, StressMarq),

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/5, 000 in TBST; overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

anti-AKT (#9272, Cell signaling),

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/1, 000 in 5 % BSA (TBST); overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

anti-IRE1α (#3294, Cell Signaling),

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/1, 000 in 5 % BSA (TBST); overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

rabbit anti-S6K (#2708, Cell Signaling) antibodies.

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/1, 000 in TBST; overnight

- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

anti-ATF6 (22799, Santa Cruz)

- Blocking: 5 % milk (TBST); 1h, RT
- 1st antibody: diluted 1/500 in TBST; overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

anti-GAPDH (#MAB374, Millipore)

- Blocking: 5 % milk (TBST); RT
- 1st antibody: diluted 1/1, 000 in TBST; overnight
- Secondary antibody: anti-mouse, diluted 1/5, 000 in 5% milk (TBST); 1 h

3.2.7. Statistics

A one-way analysis of variance analysis was used with Bonferroni's post hoc analysis for comparison between multiple groups. A student's *t*-test was used for comparison between two groups. Significance was defined as a *P* value.

3.3. Results

3.3.1. Leptin-induced signalling

By binding to the Ob-Rb long isoform of the leptin receptor, leptin activates a number of signaling pathways such as JAK2-STAT3 (Vaisse et *al*., 1996), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) (Cui *et al*., 2006), and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) (Niswender *et al*., 2001) signaling pathways. In accordance with previous observations (Vaisse et *al*., 1996; Cui *et al*., 2006; Niswender *et al*., 2001), we showed that leptin (0.5 µg/ml, 30 min) induced the activation of STAT3, Akt, and ERK in the SH-SY5Y-ObRb cells (Fig. 7). These results indicated that the leptin signal was functionally activated under the present experimental conditions.

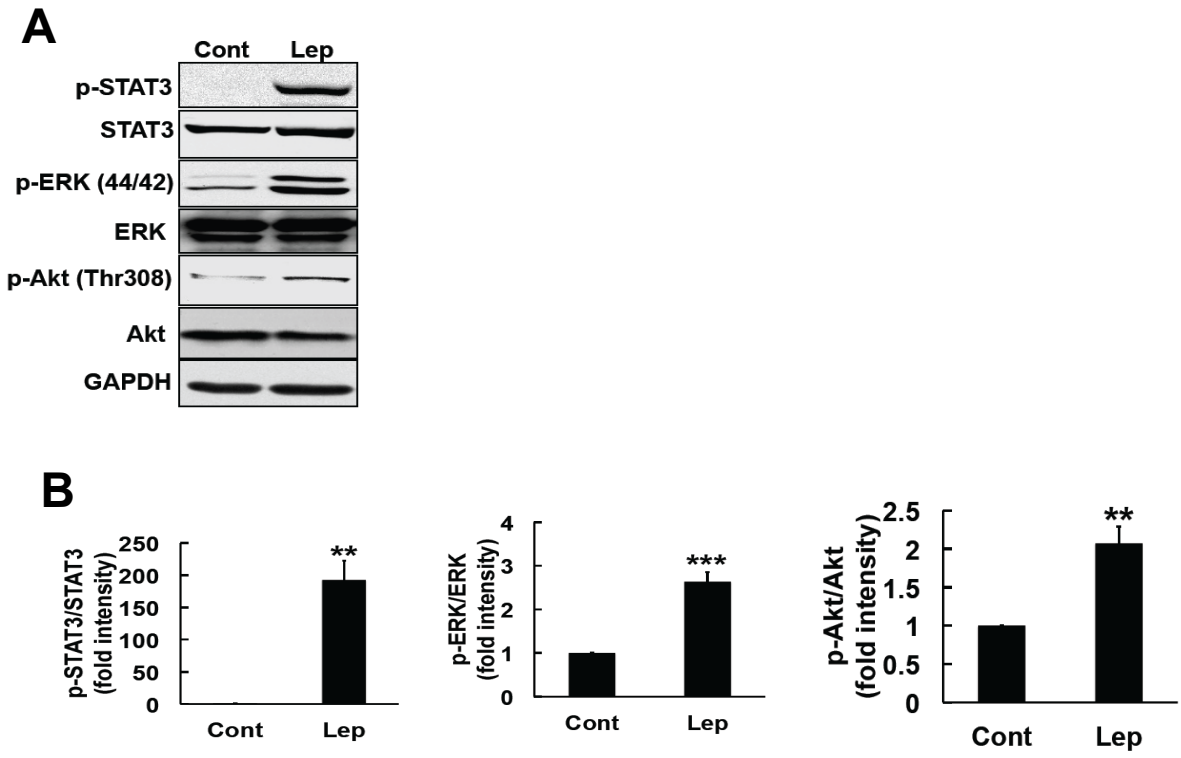


Figure 7. Leptin activated STAT3, ERK and Akt in SH-SY5Y-ObRb cells.

A. SH-SY5Y-ObRb cells were treated with leptin (0.5 μg/ml, 30 min). Phospho-STAT3 (Tyr705), STAT3, phospho-ERK1/2 (Thr202/Tyr204), ERK, phospho-Akt (Thr308), Akt, and GAPDH levels were analyzed by western blotting. Leptin induced the phosphorylation of STAT3 (Tyr705), ERK (Thr202/Tyr204), and Akt (Thr308). **B.** A densitometric analysis of phospho-STAT3 (Tyr705), phospho-ERK1/2 (Thr202/Tyr204), and phospho-Akt (Thr308) was performed using image analysis software. Data are expressed as the mean ± S.E. from 3 independent experiments (n=3). ** $P < 0.01$; *** $P < 0.001$.

3.3.2. Leptin induced GRP78 expression

GRP78, a major ER chaperone, plays protective roles against ER stress by promoting folding to prevent the aggregation of proteins (Mori, 2000). We examined whether leptin could induce the expression of GRP78 in SH-SY5Y-ObRb cells. Cells

were treated with leptin (0.5 μg/ml) for 0.5, 2, 4, and 24 h. Leptin significantly increased the expression of GRP78 after 4 and 24 h (Fig. 8A).

We then determined the mechanism of leptin-induced the expression of GRP78. The induction of GRP78 is known to be a downstream of the classical inositol-requiring enzyme 1-X-box-binding protein 1 (IRE1-XBP1) pathway. The activation of IRE1 induces spliced-XBP1 mRNA. XBP1 translated from spliced mRNA acts as a transcriptional factor for UPR-regulated genes such as the GRP78 gene (Yoshida *et al*., 2001). Thus, we investigated whether leptin could activate the phosphorylation of IRE1. Cells were treated with leptin (0.5 μg/ml) for 0.5, 2, 4, and 24 h. The ER stress inducer, tunicamycin, was used as a positive control. Leptin did not activate IRE1 at the times investigated (Fig. 8B). We at the same time investigated whether leptin could activate XBP1 splicing. Cells were treated with leptin (0.5 μg/ml) for 2, 4, and 8 h, and XBP1 mRNA expression levels were analyzed by RT-PCR. Tm was used as a positive control. The splicing of XBP1 was not detected in response to leptin (Fig. 8C). These results indicated that leptin-induced GRP78 was not mediated through the IRE1-XBP1 pathway.

We subsequently investigated whether leptin could also activate CHOP. To obtain a positive control for the expression of CHOP, SH-SY5Y-ObRb cells were treated with the ER stress inducer, thapsigargin. We successfully detected CHOP in SH-SY5Y-ObRb cells after a 4 h treatment with Tg (Fig. 8D). We then analyzed the expression of CHOP with the leptin stimulation. Leptin did not induce the expression of CHOP (Fig. 8D). This result suggested that leptin may specifically indicate the GRP78.

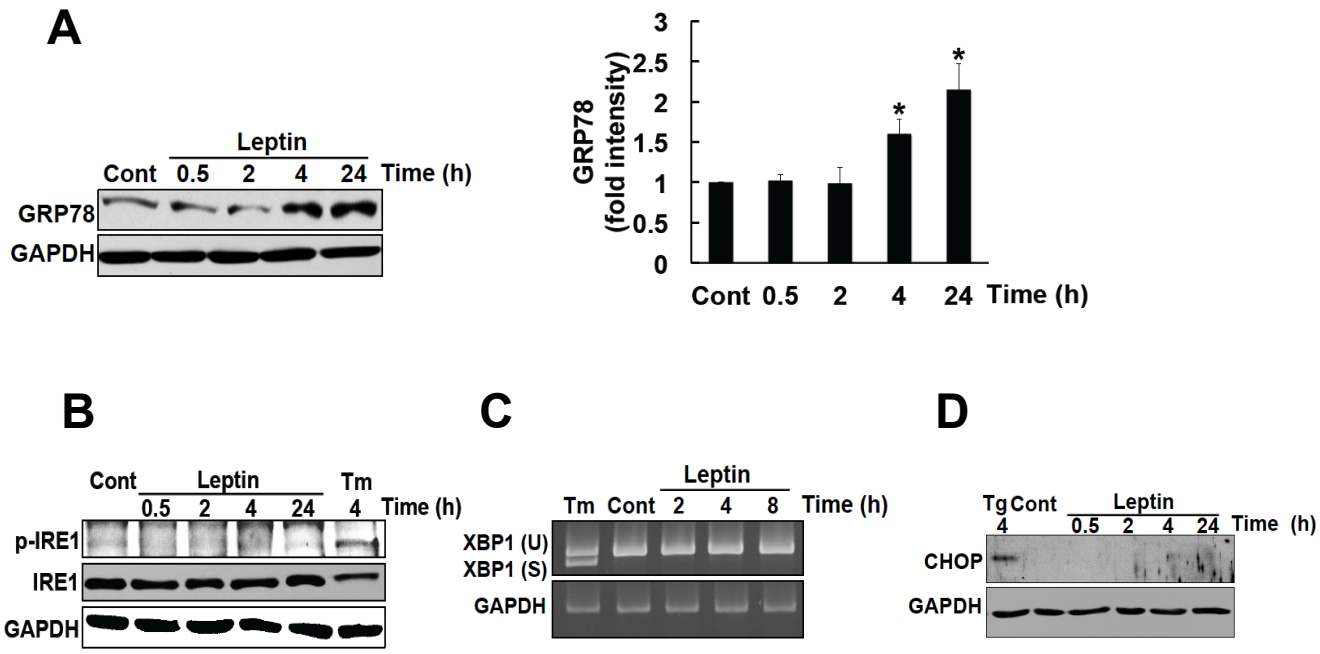


Figure 8. Leptin induced GRP78, but not CHOP or IRE1 phosphorylation.

SH-SY5Y-ObRb cells were treated with leptin (0.5 μg/ml) for 0.5, 2, 4, and 24 h. **A.** Leptin significantly increased the expression of GRP78 after 4 and 24 h. A densitometric analysis of GRP78 was performed using image analysis software. Data are expressed as the mean ± S.E. of 3 independent experiments (n = 3). * $P < 0.05$. **B.** Leptin did not induce the phosphorylation of IRE1α. Tunicamycin (Tm) (1 μg/ml, 4 h) was used as the positive control. Typical data of 3 independent experiments were shown. **C.** Leptin did not induce a spliced form of XBP1. SH-SY5Y-ObRb cells were treated with leptin (0.5 μg/ml) for 2, 4, and 8 h. Tunicamycin (Tm) (1 μg/ml, 8 h) was used as the positive control. Typical data of 3 independent experiments were shown. **D.** Cells were treated with thapsigargin (Tg) (0.2 μM, 4 h) as the positive control. Leptin did not induce the expression of CHOP. Typical data of 3 independent experiments were shown.

3.3.3. Involvement of PI3K in the leptin-induced expression of GRP78

We next examined the mechanism responsible for the leptin-induced expression

of GRP78. Based on the activation of Akt and ERK in the SH-SY5Y-ObRb model (Fig. 7A and B), we investigated whether leptin-activated Akt and ERK mediated the expression of GRP78. We used specific inhibitors of PI3K, LY294002, and MEK, PD98059. We showed the efficacy and specificity of each inhibitor by pretreating SH-SY5Y-ObRb cells with LY294002 (5 μM) and PD98059 (10 μM) for 30 min, followed by leptin. Phosphorylation levels of Akt and ERK1/2 were then analyzed. LY294002 significantly inhibited the phosphorylation of Akt. The slight inhibition of ERK phosphorylation was also observed by LY294002. This result was consistent with previous findings in which PI3K activity was responsible for activating the Raf/MEK1/ERK pathway (Wennström *et al*., 1999). On the contrary, PD98059 inhibited the phosphorylation of ERK, but not Akt (Fig. 9A). Therefore, LY294002 and PD98059 specifically inhibited the activation of PI3K and MEK. We also examined whether the leptin-mediated activation of STAT3 was dependent on Akt and ERK1/2 under the same conditions. The pretreatment the cells with LY294002 and PD98059 did not alter the leptin-mediated phosphorylation of STAT3 (Fig. 9A), indicating that the leptin-induced activation of STAT3 was independent of PI3K and MEK.

To illustrate whether attenuating the Akt and ERK pathways are responsible for the leptin-induced GRP78, SH-SY5Y-ObRb cells were pretreated with LY294002 (5 μM) and PD98059 (10 μM) for 30 min, followed by the leptin stimulation (0.5 μg/ml, 24 h) and GRP78 levels were then analyzed by western blotting. The PI3K inhibitor blocked the effects of leptin on GRP78 induction (Fig. 9B), whereas the MEK inhibitor did not. These results suggested that the leptin-induced expression of GRP78 depended on the PI3K/Akt signaling pathway.

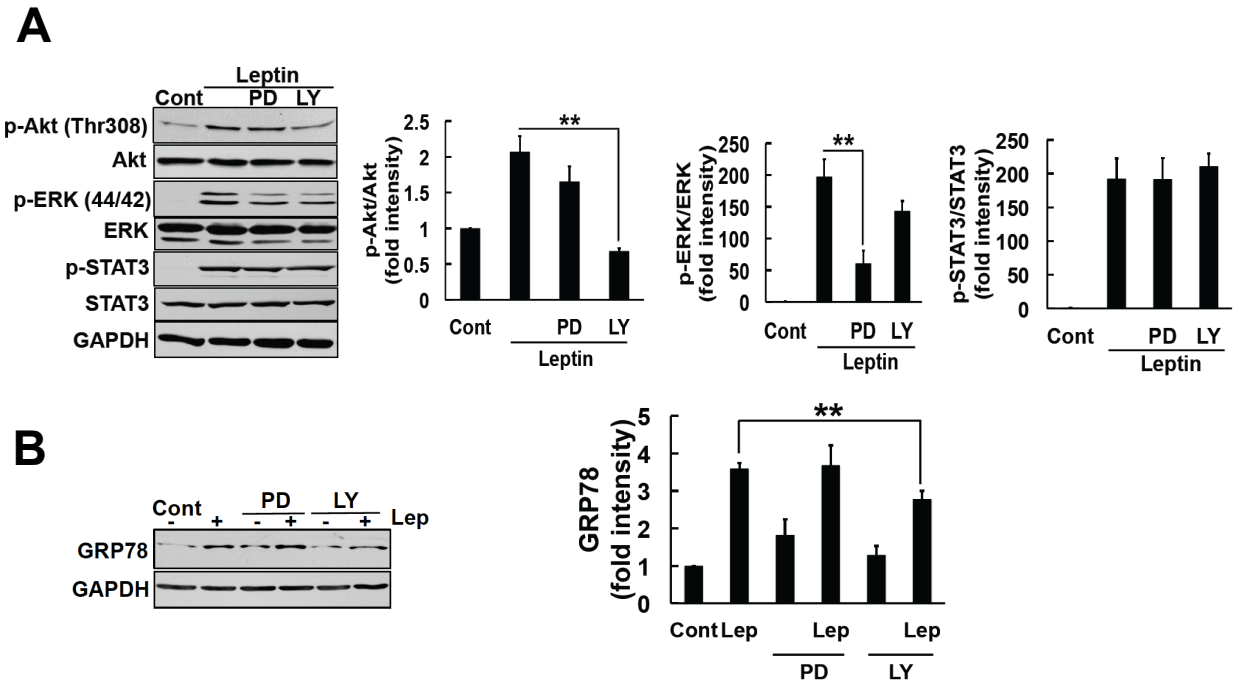


Figure 9. Involvement of PI3K in the leptin-induced GRP78 expression.

A. SH-SY5Y-ObRb cells were pretreated with PD98059 (PD; 10 μM) or LY294002 (LY; 5 μM) for 30 min followed by leptin (0.5 μg/ml, 30 min). Phospho-Akt (Thr308), Akt, phospho-ERK 1/2 (Thr202/Tyr204), ERK, phospho-STAT3 (Tyr705), STAT3, and GAPDH levels were analyzed by western blotting. A densitometric analysis of phospho-Akt (Thr308), phospho-ERK1/2 (Thr202/Tyr204), and phospho-STAT3 (Tyr705) were performed using image analysis software. The treatment with PD inhibited the phosphorylation of ERK, but not Akt. The treatment with LY inhibited the phosphorylation of Akt and also slightly inhibited that of ERK. The treatment with PD or LY did not inhibit the phosphorylation of STAT3. Data are expressed as the mean ± S.E. of 3 independent experiments (n = 3). * $P < 0.05$; ** $P < 0.01$. **B.** SH-SY5Y-ObRb cells were pretreated with PD98059 (PD; 10 μM) or LY294002 (LY; 5 μM) for 30 min followed by leptin (0.5 μg/ml, 24 h). GRP78 and GAPDH levels were analyzed by western blotting. A densitometric analysis of GRP78 was performed using image analysis software. LY prevented the effects of

leptin on the induction of GRP78 expression. Data are expressed as the mean ± S.E. of 4 independent experiments (n = 4). ** $P < 0.01$.

3.3.4. Leptin induced S6K phosphorylation through the PI3K/Akt pathway

We examined one of the downstream regulators of the PI3K/Akt pathway. The activation of Akt is known to participate in important cellular processes through the mammalian target of rapamycin (mTOR) pathway (Engelman *et al*., 2006). Therefore, we hypothesized that leptin may activate GRP78 via the Akt/mTOR signaling pathway. SH-SY5Y-ObRb cells were treated with leptin (0.5 μg/ml, 30 min) and a downstream of mTOR, S6K, was analyzed by western blotting. Consistent with previous findings (Maya-Monteiro *et al*., 2008), we demonstrated that leptin increased phosphorylation of S6K (Thr389) levels in SH-SY5Y-ObRb cells (Fig. 10). We next examined the effects of LY294002 on S6K phosphorylation. Cells were pretreated with LY294002 (5 μM, 30 min) followed by leptin (0.5 μg/ml, 30 min) and S6K phosphorylation levels were analyzed by western blotting. The results showed that the phosphorylation of S6K was blocked by LY294002 (Fig. 10). Therefore, these results indicated that S6K activation was located downstream of PI3K under leptin-induced signaling in SH-SY5Y-ObRb cells.

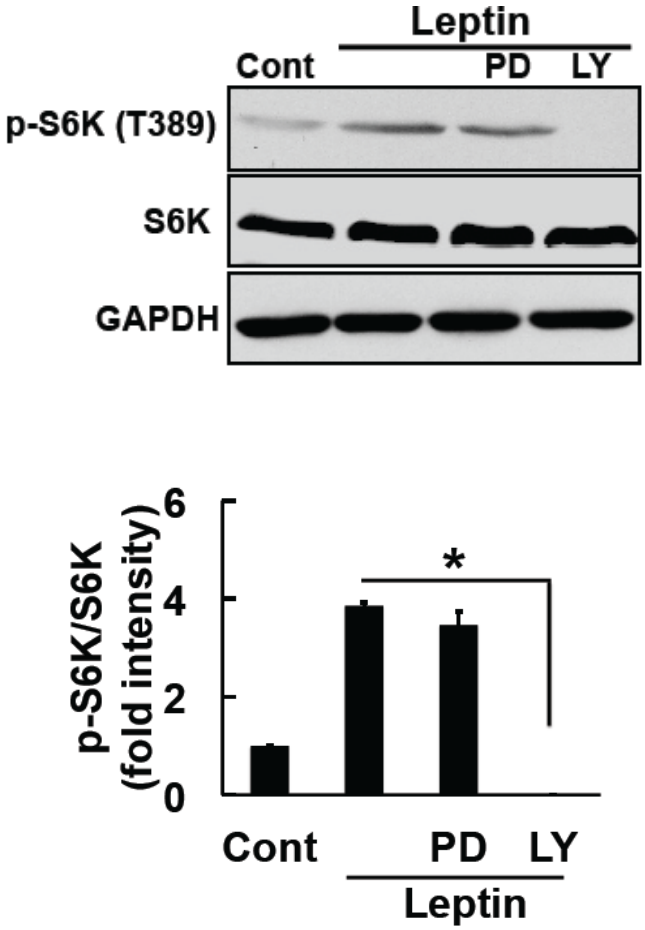


Figure 10. Leptin induced the phosphorylation of S6K through the PI3K/Akt pathway in SH-SY5Y-ObRb cells.

SH-SY5Y-ObRb cells were pretreated with PD98059 (PD; 10 μM) or LY294002 (LY; 5 μM) for 30 min followed by leptin (0.5 μg/ml, 30 min). Phospho-S6K (p-S6K:Thr389), S6K, and GAPDH levels were analyzed by western blotting. A densitometric analysis of phospho-S6K (p-S6K:Thr389) was done using image analysis software. The leptin-induced phosphorylation of S6K was blocked by LY294002. Data are expressed as the mean ± S.E. of 3 independent experiments (n = 3). * $P < 0.05$.

3.3.5. Leptin-induced GRP78 expression was mediated through the mTOR

pathway

To further confirm our results that the leptin-induced expression of GRP78 was dependent on the mTOR pathway, we used mTOR-specific inhibitor, rapamycin. We first checked whether rapamycin could inhibit the phosphorylation of S6K. SH-SY5Y-ObRb cells were pretreated with rapamycin (10 nM) for 30 min followed by leptin (0.5 μg/ml). We found that rapamycin markedly inhibited the leptin-induced phosphorylation of S6K (Fig. 11A).

We then validated the contribution of the mTOR pathway to the expression of GRP78. Cells were treated with rapamycin (10 nM) and leptin (0.5 μg/ml) for 24 h. The results showed that rapamycin significantly blocked the leptin-induced expression of GRP78 (Fig. 11B). These results suggested that leptin may activate the mTOR pathway, leading to an increase in GRP78 levels.

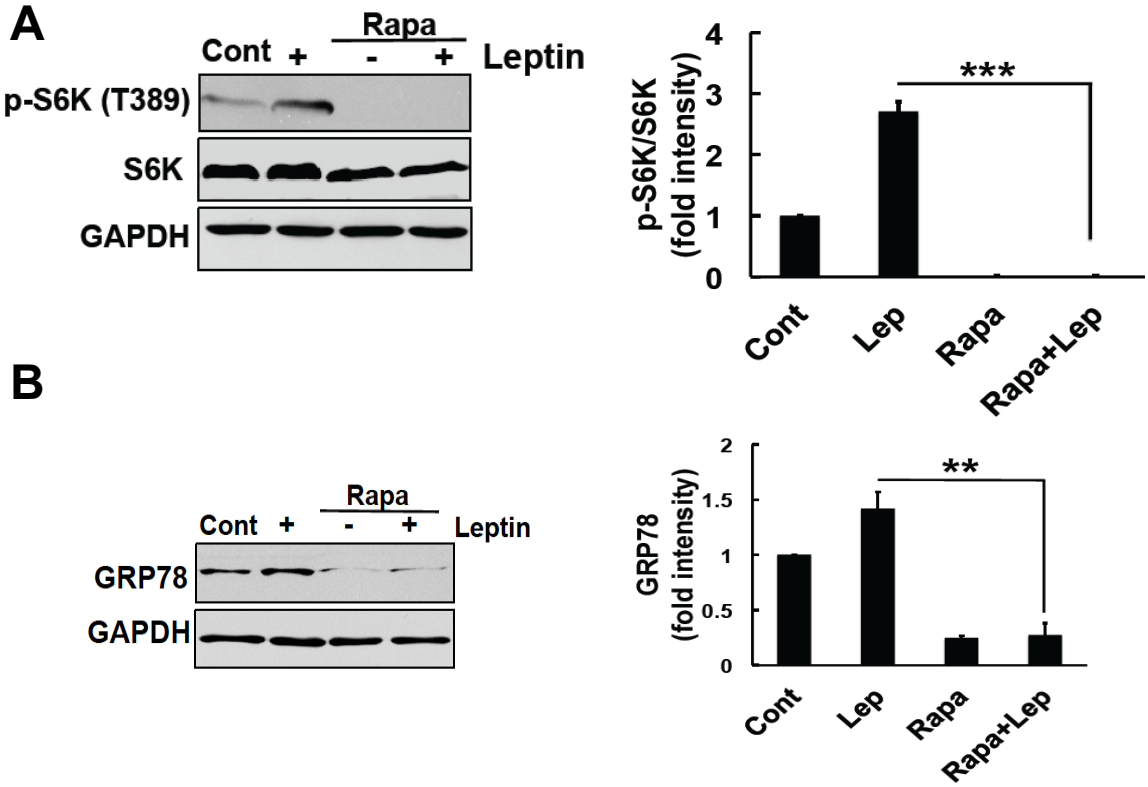


Figure 11. The leptin-induced expression of GRP78 was mediated through the mTOR pathway in SH-SY5Y-ObRb cells.

A. SH-SY5Y-ObRb cells were pretreated with rapamycin (Rapa; 10 nM) followed by leptin (0.5 μg/ml, 30 min). Phospho-S6K (p-S6K:Thr389), S6K, and GAPDH levels were analyzed by western blotting. A densitometric analysis of phospho-S6K level was done using image analysis software. Rapamycin drastically inhibited leptin-induced S6K phosphorylation. Data are expressed as the mean ± S.E. of 3 independent experiments (n = 3). *** $P < 0.001$. **B.** SH-SY5Y-ObRb cells were treated with rapamycin (Rapa; 10 nM) and stimulated with leptin (0.5 μg/ml, 24 h). GRP78 and GAPDH levels were analyzed by western blotting. A densitometric analysis of GRP78 was performed using image analysis software. Rapamycin significantly blocked the leptin-induced expression of GRP78. Data are expressed as the mean ± S.E. of 3 independent experiments (n = 3). ** $P < 0.01$.

3.3.6. Involvement of the PI3K/mTOR pathway in the leptin-induced proliferation

We performed a cell proliferation assay to determine the physiological consequences of the effects of leptin. Since, we showed that the PI3K-mTOR pathway played an important role in the induction of GRP78, we next investigated the effects of the PI3K inhibitor and mTOR inhibitor on the leptin-induced proliferation. Forty-eight hours after seeding, SH-SY5Y-ObRb cells were washed with serum-free medium and stimulated with LY294002 (5 μM) or rapamycin (10 nM) for 30 min followed by leptin for 24 h. Cell growth was measured using the WST-1 assay. As shown in Figure 12, both LY294002 and rapamycin reduced leptin-induced proliferation. These results signified that activation of the Akt/mTOR pathway mediated the cell proliferative effects of leptin in SH-SY5Y-ObRb cells.

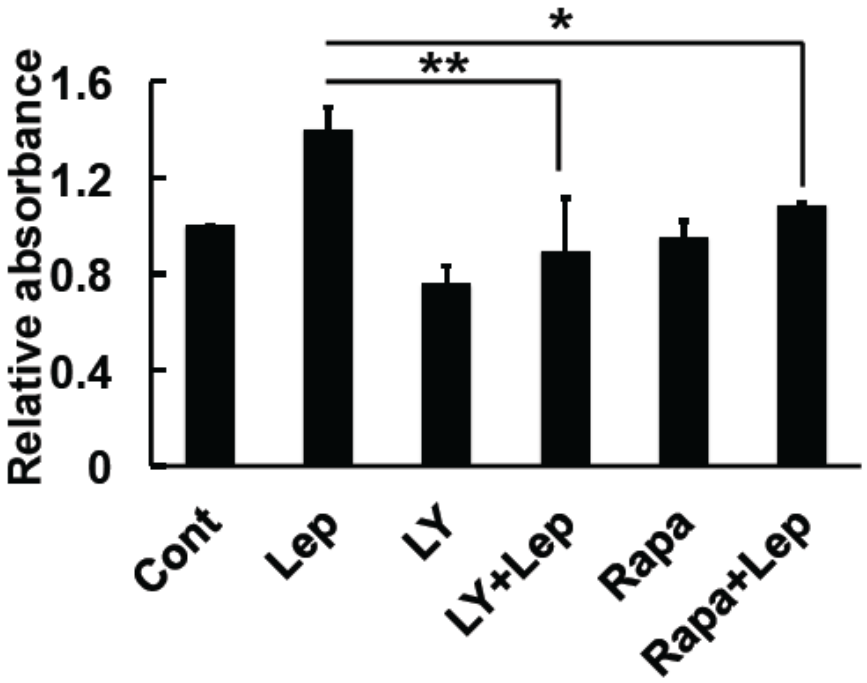


Figure 12. Involvement of the PI3K/mTOR pathway on the leptin-induced proliferation of SH-SY5Y-ObRb cells.

SH-SY5Y-ObRb cells were pretreated with LY294002 (LY; 5 μM) or rapamycin (Rapa; 10 nM) for 30 min and then stimulated with leptin (0.5 μg/ml) for 24 h. Cells were incubated with the WST-1 reagent for 4 h and then analyzed for optical density. LY294002 and rapamycin reduced leptin-induced proliferation. Data are expressed as the mean ± S.E. of 4 independent experiments (n = 4). * $P < 0.05$, ** $P < 0.01$.

3.3.7. Leptin protected against ER stress-induced cell death

To evaluate the physiological outcome of leptin against ER stress, we examined its effects on ER stress-induced cell death. SH-SY5Y-ObRb cells were pre-treated with leptin (0.5 μg/ml, 8 h) followed by ER stress inducers (tunicamycin, 0.3 μg/ml, 48 h). Cell death was then measured using the LDH assay. Treatment the cells with tunicamycin caused cell death and this was significantly lessened by leptin (Fig. 13). Therefore, these results suggested that leptin may be able to attenuate ER stress-induced cell death.

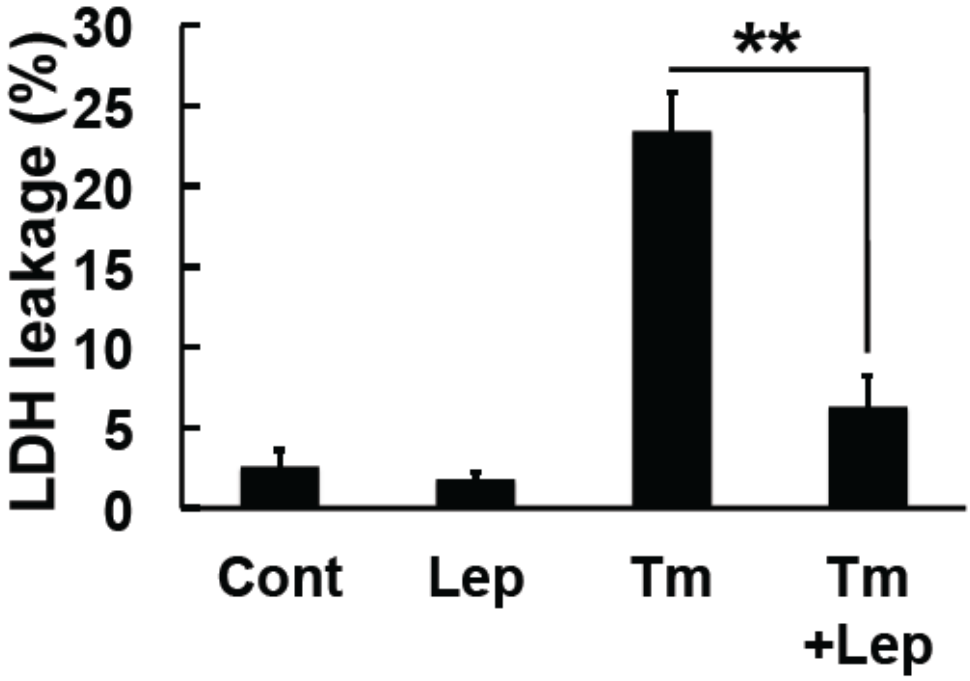


Figure 13. Leptin ameliorated ER stress-induced cell death in SH-SY5Y-ObRb cells.

Cells were pretreated with leptin (0.5 μg/ml) for 8 h and followed by tunicamycin (Tm, 0.3 μg/ml) for another 48 h. Lactate dehydrogenase (LDH) activity was measured as the indicator of cytotoxicity. Data are expressed as the mean ± S.E. of 4 independent experiments (n = 4). ** $P < 0.01$.

3.4. Discussion

We found that leptin induces GRP78 in neuronal cells. Leptin-induced GRP78 induction was not mediated through its classical activation pathway, IRE1-XBP1 pathway, as we could not neither detect an increase in IRE1 phosphorylation nor spliced XBP1 in leptin-treated cells (Fig. 8B, C). Instead of the IRE1-XBP1, we showed that the leptin-induced expression of GRP78 was mediated through the PI3K-mTOR pathway, which was confirmed by the inhibition of leptin-induced GRP78

expression by the PI3K inhibitor, LY294002, and mTOR inhibitor, rapamycin (Fig. 9B and 11B).

GRP78 is a key chaperone protein that controls ER homeostasis by enhancing the folding and assembly of proteins (Gidalevitz *et al*., 2013; Ni *et al*., 2007). In the present study, we showed that leptin itself could induce GRP78 but not a pro-apoptotic marker, CHOP, in neuronal cells (Fig. 8). ER stress was shown to increase in obesity (Kawasaki *et al*., 2012; Cnop *et al*., 2012; Jiao *et al*., 2011). Previously, our group and others reported that ER stress caused leptin resistance (Hosoi *et al*., 2008; Ozcan *et al*., 2009; Won *et al*., 2009). Based on this, we speculated the physiological role of leptin-induced GRP78 expression as follows: In the early phase of a stressed condition when leptin resistance has not yet developed, leptin itself may protect against ER stress by inducing the expression of GRP78 because we found that leptin can protect against ER stress-induced cell death (Fig. 13). However, resistance may develop when ER stress is severe and prolonged, which leptin cannot afford (Hosoi *et al*., 2008; Ozcan *et al*., 2009).

Part 4: Insulin enhanced leptin-induced STAT3 signaling by inducing GRP78

4.1. Introduction

The convergence in the regulation of glucose and energy homeostasis has been reported between leptin and insulin. However, the effects of insulin on leptin's actions in the central nervous system (CNS) remain to be clearly understood. Insulin was known to induce GRP78 (Inageda, 2010). Based on this, we aimed to investigate the function of GRP78 in leptin signaling in insulin-treated neuronal cells.

4.2. Materials and methods

4.2.1. Reagents

Human insulin (Humulin®R) was purchased from Eli Lilly and Company (IN).

4.2.2. Cell culture

As described in 2.2.3. HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37°C in humidified 5% CO_2/95% air.

4.2.3. RNAi Experiment

The transient transfection of siRNA was conducted in SH-SY5Y-ObRb cells and HEK293-ObRb cells. Lipofectamine RNAiMAX (Life Technologies) was used to transfect siRNA according to the manufacturer's guidelines. Opti-MEM medium was used for transfections. We used the following siRNA in the RNAi experiments: Silencer® Select Pre-designed (Inventoried) siRNA (Life Technologies, siRNA ID

for GRP78: s6980) and Silencer® Select Negative Control siRNA #1 (Life Technologies). The final concentrations of siRNA were 2 and 5 nM. Cells were harvested 72-96 h after transfections.

4.2.4. Plasmid and transfections

pcDNA3.1(+)-GRP78/Bip was a gift from Richard C. Austin (Addgene plasmid # 32701) (Werstuck, G.H. *et al*. 2006). A mock plasmid was used as a negative control. Human HEK293-ObRb cells were transfected using the calcium phosphate transfection method. A total of 0.5 μg of DNA was used per 35 mm plate. 2xHEPES buffered saline (2xHBS) solution (16.4 g NaCl, 11.9 g HEPES, and 0.54 g Na_2HPO_4, per liter, at pH 7.15) was used to precipitate calcium phosphate/DNA. Briefly, 3 h prior to transfection, we changed to fresh medium and a solution containing the plasmid and 2.5 mM $CaCl_2$ was added to 2xHBS solution. Following a 30 min incubation at room temperature, the calcium phosphate-plasmid suspension solution was added dropwise to the culture dish. Eight hours after the incubation, culture medium was changed to DMEM containing antibiotics. Cells were harvested 48 h after transfections.

4.2.5. Immunoprecipitation and immunoblotting

pEFBOS-OBR-Long-MyC, pcDNA3.1(+)-GRP78/Bip and a mock plasmids (a negative control) were transfected in HEK293T cells using the calcium phosphate transfection method **(**as described in 4.2.4).

Myc-ObRb was immunoprecipitated from HEK293T cells after lysis in modified RIPA buffer (50 mM Tris pH 7.4, 1% Triton X-100, 1mM EDTA pH 8.0, 0.2% sodium dodecylsulfate, 0.2% sodium deoxycholate, 1 mM Na_3VO_4, 20 mM NaF, 10

μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Supernatant was incubated with anti-Myc-tag mAb (MBL, M192-3) or mouse IgG (MBL, M077-3) for 1 h (15 rotations/min, at 4^0C), followed by incubation with Protein G Magnetic Beads (Life Technologies) for 10 min (15 rotations/min, at 4^0C). The antibody-coupled Dynabeads Protein G was washed 3 times with lysis buffer. The immunopricipitates were then treated with Laemmli containing urea (final 8M) and DTT (final 100 mM) analyzed by western blotting.

4.2.6. Western blotting analysis

As described in 1.2.5. Additional antibodies used:

anti-GRP78 (GL-19) (G8918, Sigma-Aldrich)

- Blocking: 5 % milk (TBST); 1 h, RT
- 1st antibody: diluted 1/3, 000 in TBST; overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 5% milk (TBST); 1 h

anti-Myc-tag mAb (M192-3, MBL)

- blocking: 3 % milk (PBST); 1h, RT
- 1st antibody: diluted 1/5, 000 in TBST; five overnight
- Secondary antibody: anti-rabbit, diluted 1/5, 000 in 3% milk (PBST); 1 h

4.2.7. Statistics

A one-way analysis of variance analysis was used with Bonferroni's post hoc analysis for comparison between multiple groups. A paired *t*-test was used for comparison between two groups. Significance was defined as a *P* value.

4.3. Results

4.3.1. Insulin induced the phosphorylation of Akt and S6K

To begin with, we first analyzed insulin-induced signals in our experimental conditions. Insulin activates PI3K and mTOR pathways resulted in its downstream activation of Akt and S6K (Czech *et al* 1999; Takano *et al*., 2001). Accordingly, we showed that insulin phosphorylated Akt and S6K in SH-SY5Y-ObRb cells (Fig. 14). Thus, insulin signaling was functionally activated in the SH-SY5Y-ObRb cell line.

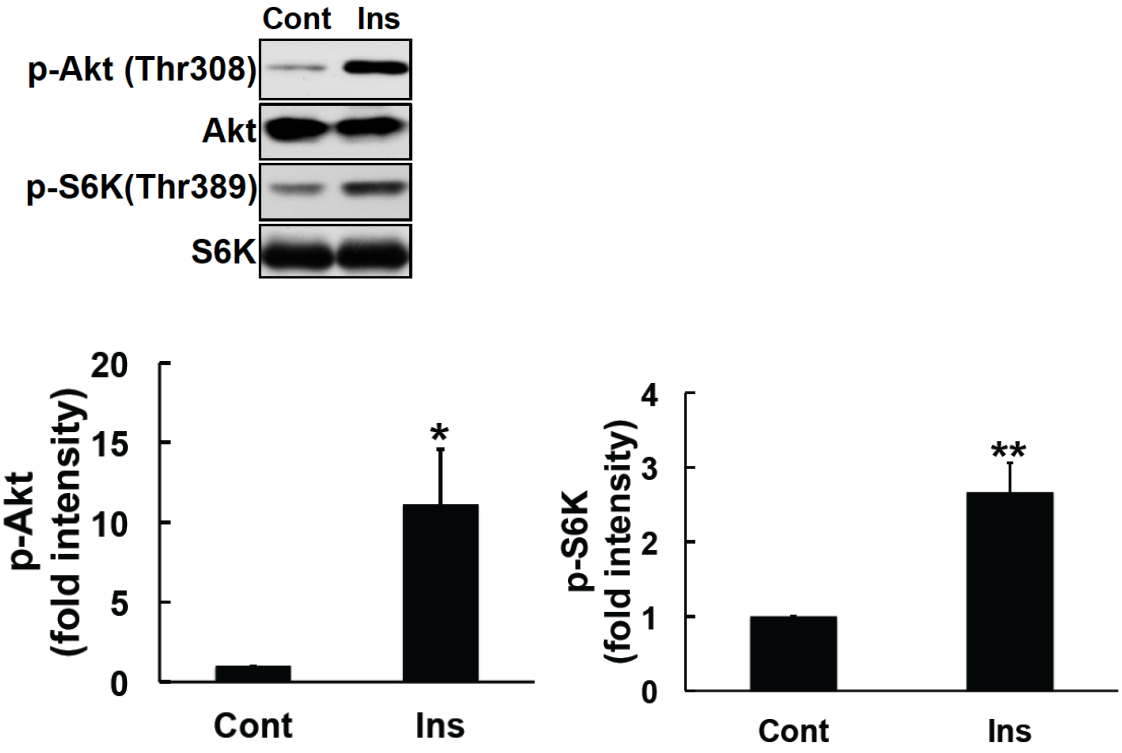


Figure 14. Insulin induced Akt and S6K activation in SH-SY5Y-ObRb cells. SH-SY5Y-ObRb cells were exposed to serum-free medium for 20 h and treated with insulin (300 nM) for 4 h. Phospho-Akt (p-Akt:Thr308), Akt, phospho-S6K (p-S6K:Thr389), and S6K were analyzed by western blotting. Insulin increased the phosphorylation of Akt (Thr308) and S6K (Thr389). A densitometric analysis of phospho-Akt (Thr308) and phospho-S6K (Thr389) was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n=4). * $P < 0.05$; ** $P < 0.01$.

4.3.2. Insulin enhanced leptin-induced STAT3 activation

Anti-obesity effects of leptin carry out through the JAK2-STAT3 signaling pathway (Vaisse *et al*., 1996). Insulin and leptin target the same hypothalamic area in order to suppress eating behavior (Brüning *et al*., 2000); thus, an investigation of the interaction between leptin and insulin may provide a fundamental understanding of obesity and its related diseases. In the current study, we examined the effects of leptin in the presence or absence of insulin. SH-SY5Y-ObRb cells were changed to a serum-free condition 20 h prior to the exposure of cells to insulin (10 and 300 nM, 4 h). Leptin was (0.03 μg/ml, 15 min) then stimulated. We showed that the co-stimulation with insulin and leptin enhanced leptin induced-STAT3 comparing to the leptin treatment alone (Fig. 15A).

At the same time, we also investigated the effect of insulin alone on STAT3 phosphorylation. SH-SY5Y-ObRb cells were exposed to serum-free medium for 24 h. Different concentration of insulin (10, 30, 100 and 300 nM) was added during the last 15 min. As a result, treatment with insulin alone did not induce phosphorylation of STAT3 (Fig. 15B).

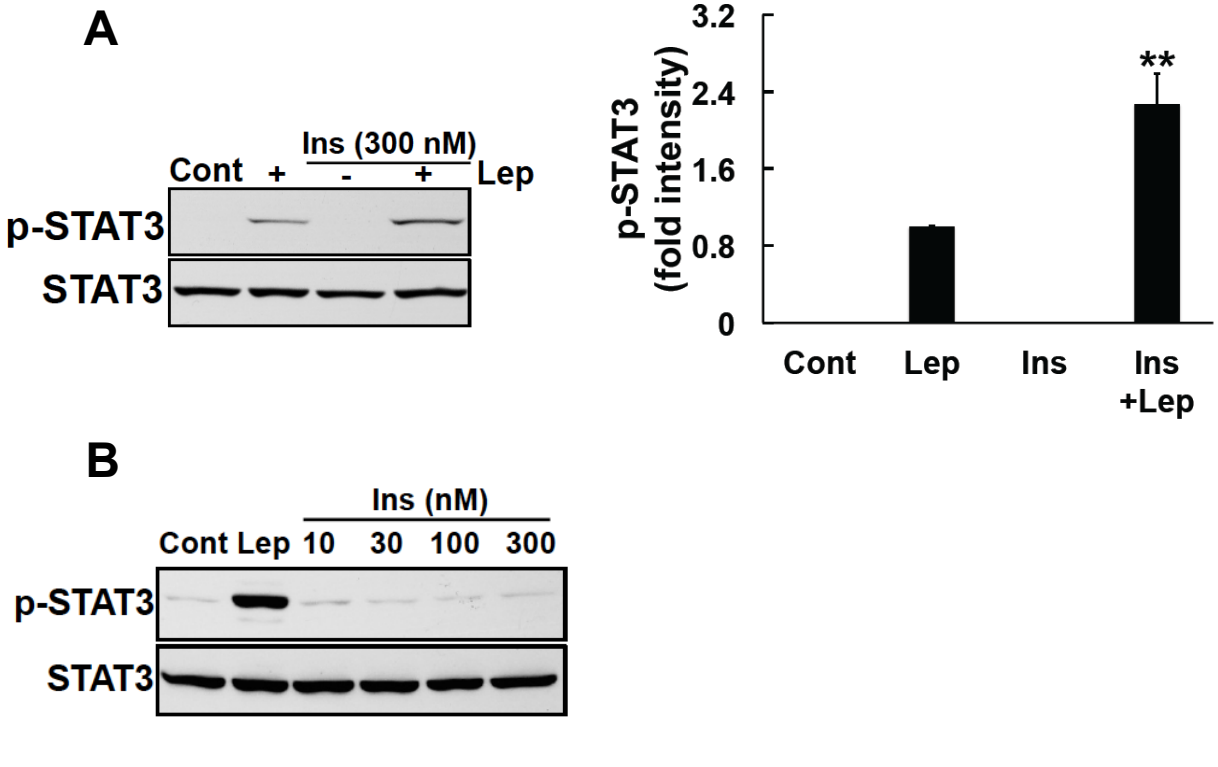


Figure 15. Insulin enhanced leptin-induced STAT3 phosphorylation in SH-SY5Y-ObRb cells.

A. SH-SY5Y-ObRb cells were exposed to serum-free medium for 20 h. Cells were treated with insulin (300 nM) for 4 h, followed by leptin (0.03 μg/ml, 15 min). Phospho-STAT3 (p-STAT3:Tyr705) and STAT3 levels were analyzed by Western blotting. A co-stimulation with insulin and leptin enhanced the leptin-induced phosphorylation of STAT3. A densitometric analysis of phospho-STAT3 (p-STAT3:Tyr705) was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n=4). ** $P < 0.01$. **B.** SH-SY5Y-ObRb cells were exposed to serum-free medium for 24 h. Cells were then treated with insulin (10, 30, 100 and 300 nM). Leptin (0.03 μg/ml, 15 min) was used as a positive control. After 15 min, phospho-STAT3 (p-STAT3:Tyr705) and STAT3 levels were analyzed by western blotting. Insulin did not induce the phosphorylation of STAT3 in SH-SY5Y-ObRb cells. Typical data of 3 independent experiments were shown.

4.3.3. Insulin induced GRP78

Both leptin and insulin activate PI3K (Carvalheira *et al.,* 2005; Morton *et al*., 2005). The PI3K-mTOR pathway is one of the upstream pathways involved in the induction of GRP78 (Pfaffenbach *et al*., 2012). Of note, insulin was previously reported to increase GRP78 levels via the PI3K-ATF4 pathway (Inageda, 2010). These findings suggest that insulin-induced GRP78 may influence the anti-obesity effects of leptin. Therefore, we examined the induction of GRP78 by insulin under our experimental conditions. SH-SY5Y-ObRb cells were exposed to serum-free medium for 20 h and stimulated with insulin (300 nM) for another 4 h. In accordance

with previous finding (Inageda, 2010), we showed that insulin induced GRP78 in the SH-SY5Y-ObRb cell model (Fig. 16).

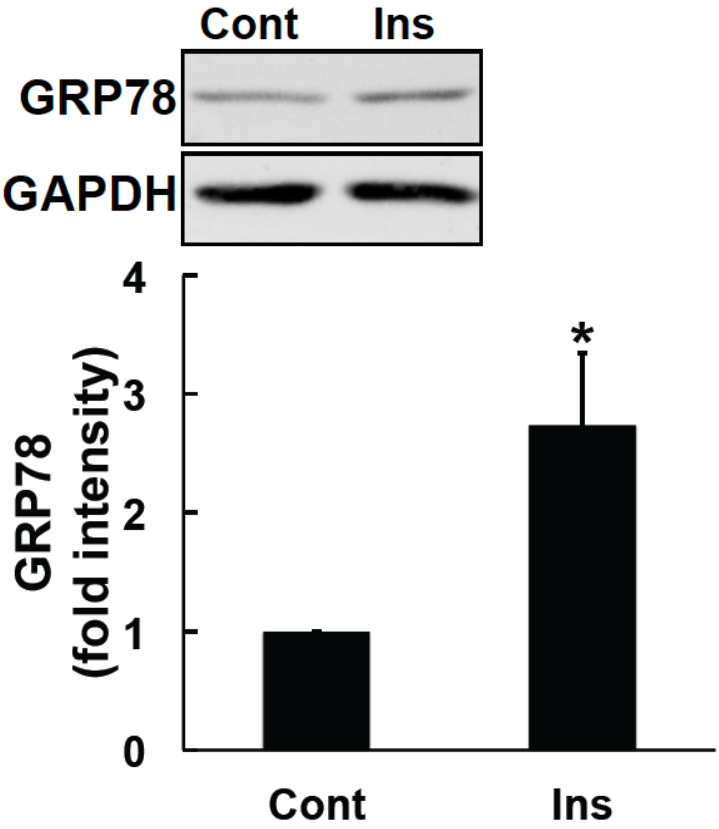


Figure 16. Insulin induced GRP78 expression in SH-SY5Y-ObRb cells.

SH-SY5Y-ObRb cells were exposed to serum-free medium for 20 h. Cells were then treated with insulin (300 nM) for 4 h. GRP78 and GAPDH levels were analyzed by western blotting. Insulin induced GRP78 expression levels. A densitometric analysis of GRP78 was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n=4). * $P < 0.05$.

4.3.4. Involvement of GRP78 in insulin-induced enhancements in leptin's actions

We next evaluated whether insulin-induced GRP78 is involved in enhancing leptin-induced STAT3 activation. To do this, we knocked down GRP78 in insulin-treated cells and analyzed leptin-induced STAT3 phosphorylation. Insulin failed to

enhance leptin-induced STAT3 activation in GRP78-knocked down cells (Fig. 17). Taken together, these results suggest that insulin enhances leptin-induced STAT3 phosphorylation via the induction of GRP78.

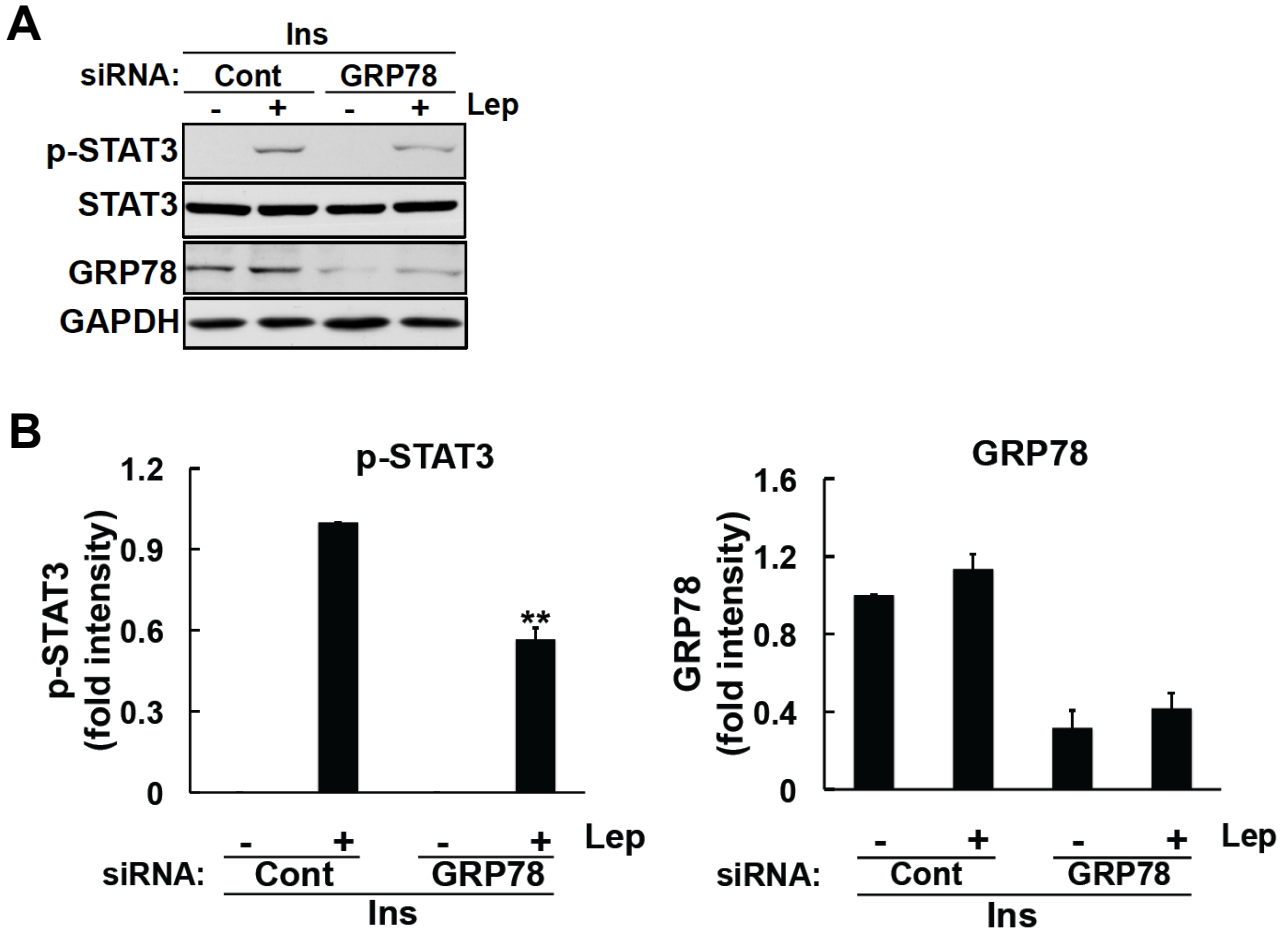


Figure 17. Insulin-mediated enhancements in STAT3 signaling were mediated through GRP78 in SH-SY5Y-ObRb cells.

SH-SY5Y-ObRb cells were transfected with siRNA (5 nM) for 72 h. Seventy-two hours after the transfection, medium was switched to serum-free medium for another 20 h. Cells were treated with insulin (300 nM) for 4 h followed by leptin (0.03 μg/ml, 15 min). Phospho-STAT3 (Tyr705), STAT3, GRP78, and GAPDH levels were analyzed by Western blotting. **A.** Leptin-induced STAT3 phosphorylation in insulin-treated cells was ameliorated in GRP78-knocked down cells. **B.** A densitometric

analysis of phospho-STAT3 (p-STAT3:Tyr705) and GRP78 was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n=4). ** $P < 0.01$.

4.3.5. Knocking down of GRP78 inhibited leptin-induced STAT3 in HEK293-ObRb cells

We then examined the role of GRP78 in leptin-induced STAT3 phosphorylation. To achieve this, we performed GRP78 knockdown experiments in HEK293-ObRb cells. Cells were transfected with GRP78 siRNA (2 nM) for 72 h. The expression of GRP78 was subjected to a western blotting analysis as an indicator of transfection efficacy. We confirmed that GRP78 specific siRNA efficiently inhibited its expression in HEK293-ObRb cells (Fig. 18). Under these conditions, we found that the knockdown of GRP78 significantly reduced the leptin-induced phosphorylation of STAT3 in the HEK293-ObRb cell line (Fig. 18).

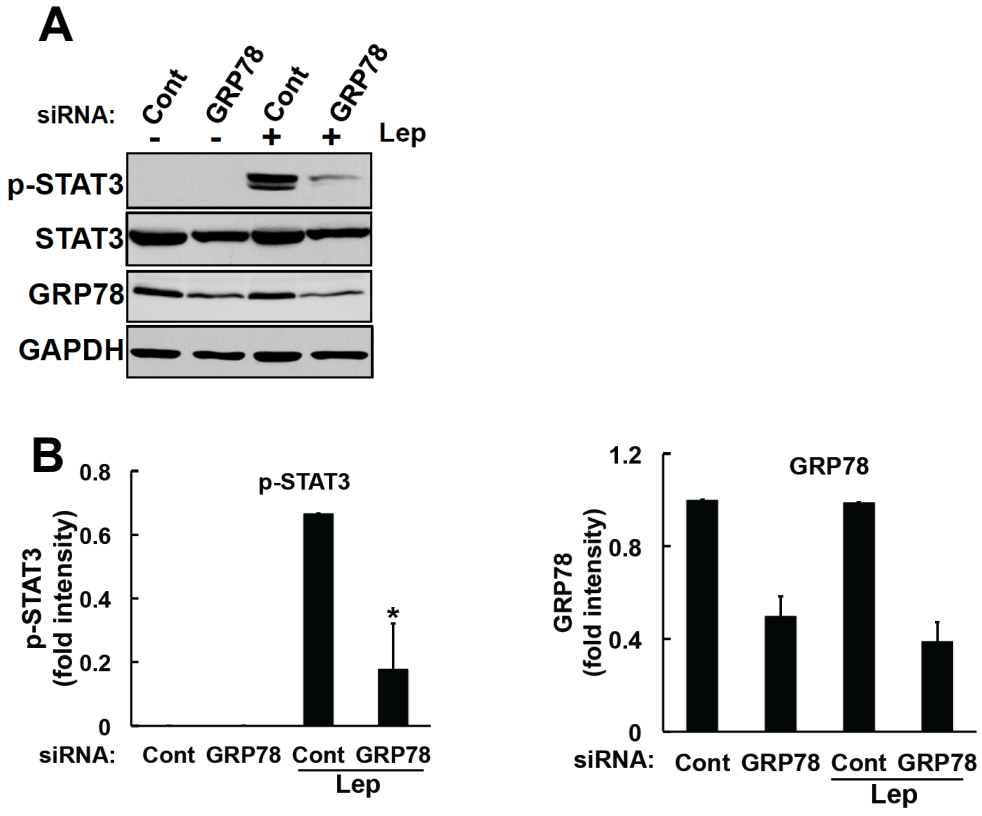


Figure 18. Leptin-induced STAT3 phosphorylation was inhibited by the knockdown of GRP78 in HEK293-ObRb cells.

HEK293-ObRb cells were transfected with siRNA (2 nM) for 72 h. Seventy-two hours after the transfection, cells were treated with leptin (0.5 μg/ml) for 15 min. Phospho-STAT3 (Tyr705), STAT3, GRP78, and GAPDH levels were analyzed by western blotting. **A**. Leptin-induced STAT3 phosphorylation was inhibited in GRP78-knocked down cells. **B.** A densitometric analysis of phospho-STAT3 (Tyr705) and GRP78 was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n=4). * $P < 0.05$.

4.3.6. Knocking down of GRP78 inhibited leptin-induced STAT3 in SH-SY5Y-ObRb cells

We simultaneously investigated the effect of GRP78 knockdown on leptin-induced signal in SH-SY5Y-ObRb neuronal cellular model. SH-SY5Y-ObRb cells were transfected with GRP78 siRNA (5 nM) for 72 h. Seventy-two hours after the transfection, medium was switched to serum-free medium for another 24 h followed by leptin stimulation (0.03 μg/ml, 15 min). GRP78 expression was inhibited by GRP78 specific siRNA (Fig. 19). We found that leptin-induced STAT3 phosphorylation was also significantly attenuated in GRP78-knockdown- SH-SY5Y-ObRb cells (Fig. 19).

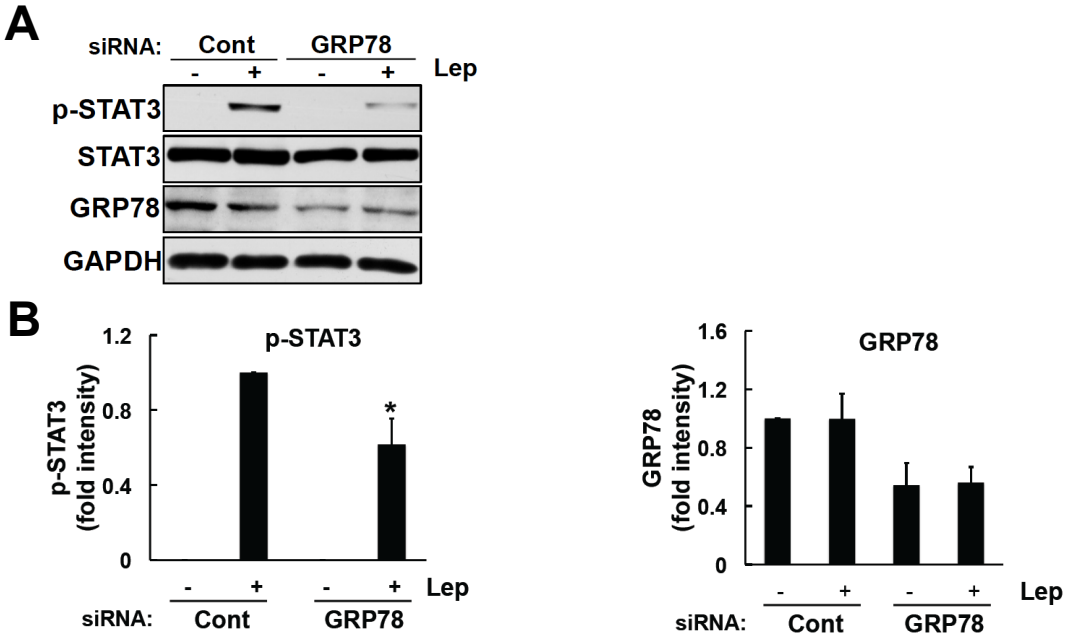


Figure 19. Leptin-induced STAT3 phosphorylation was inhibited by the knockdown of GRP78 in SH-SY5Y-ObRb cells.

SH-SY5Y-ObRb cells were transfected with control or GRP78 siRNAs (5 nM) for 72 h. Seventy-two hours after the transfection, medium was changed to serum-free medium for another 24 h. Cells were treated with leptin (0.03 μg/ml, 15 min). Phospho-STAT3 (p-STAT3:Tyr705), STAT3, GRP78, and GAPDH levels were analyzed by western blotting. **A.** Leptin-induced STAT3 phosphorylation was attenuated in GRP78-knocked down cells. **B.** A densitometric analysis of phospho-STAT3 (p-STAT3:Tyr705) and GRP78 was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n=4). * $P < 0.05$.

4.3.7. Overexpression of GRP78 enhanced leptin-induced STAT3 in HEK293-ObRb cells

To elucidate the role of GRP78 in leptin-induced STAT3 signaling, we then overexpressed GRP78 and analyzed leptin-induced signaling. The GRP78 construct was transfected into HEK293-ObRb cells for 48 h and the expression of GRP78 was then analyzed. We showed that the transfection of GRP78 increased its levels in HEK293-ObRb cells (Fig. 20). We then stimulated this cellular model with leptin and analyzed phosphorylation of STAT3. As shown in Figure 20, STAT3 phosphorylation was significantly higher in cells overexpressing GRP78 than in mock-transfected cells. These results suggest that GRP78 plays an important role in the activation of leptin-induced STAT3 signaling.

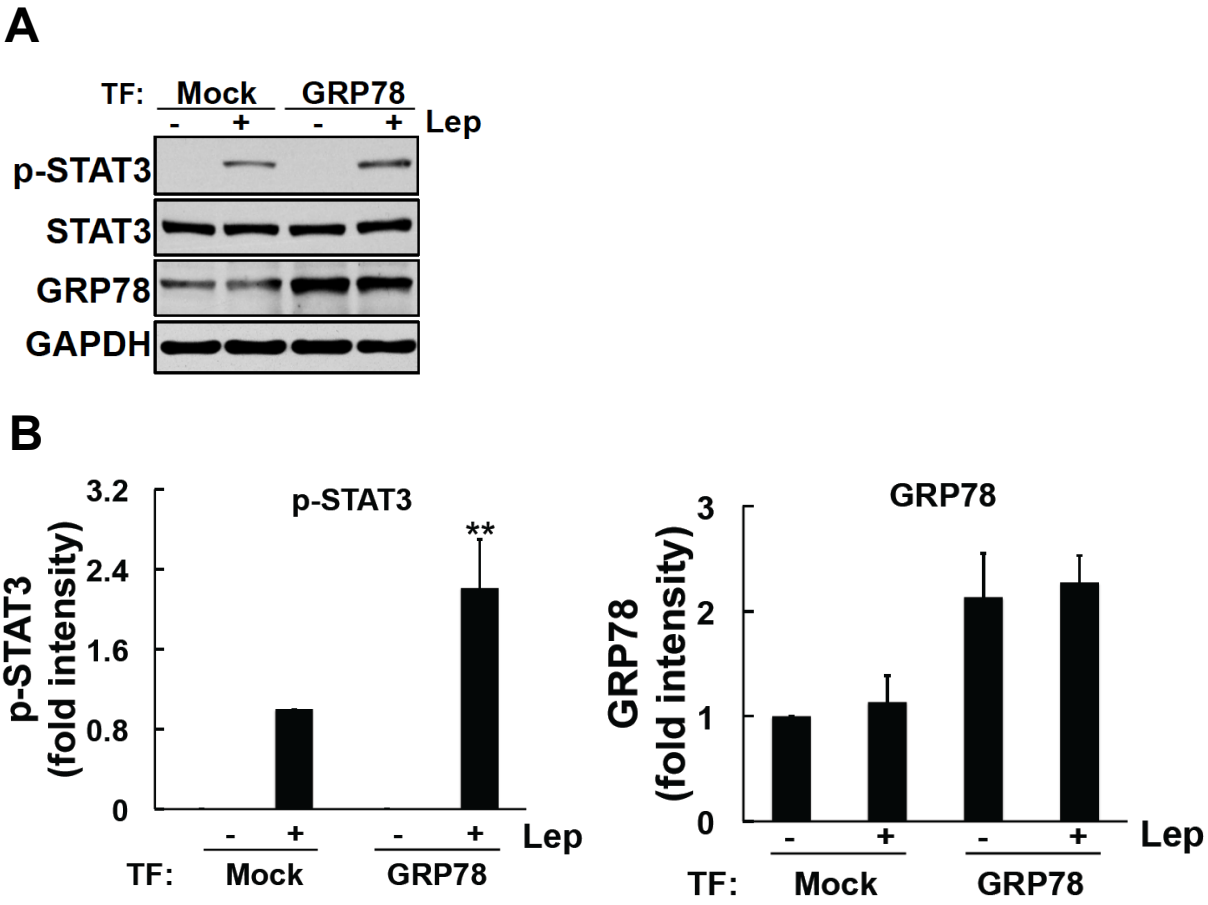


Figure 20. Overexpression of GRP78 enhanced leptin-induced STAT3 phosphorylation in HEK293-ObRb cells.

HEK293-ObRb cells were transfected with a GRP78 construct. Forty-eight hours after the transfection, cells were treated with leptin (0.03 μg/ml) for 15 min. Phospho-

STAT3 (p-STAT3:Tyr705), STAT3, GRP78, and GAPDH levels were analyzed by western blotting. **A.** GRP78 construct enhanced leptin-induced STAT3 activation. **B.** A densitometric analysis of phospho-STAT3 (p-STAT3:Tyr705) and GRP78 was performed using image analysis software. Data are expressed as the mean ± S.E. of 4 independent experiments (n=4). ** $P < 0.01$.

4.3.8. Clarification the mechanisms of GRP78-modulated leptin signaling

Having found the important role of GRP78 on leptin-induced signals, as signified by the inhibition of leptin-induced phosphorylation of STAT3 in GRP78 knocked down cells and the enhancement of leptin-induced STAT3 phosphorylation in overexpression of GRP78, we further studied the mechanism of which GRP78 mediated modulation on leptin signaling.

We hypothesized the interaction between GRP78 and the long isoform of leptin receptor (ObRb). To achieve this, we performed an immunoprecipitation assay. GRP78 and Myc-ObRb constructs were transfected in HEK293T cells. Seventy-two hours after the transfection, proteins were extracted and subjected to immunoprecipitation with anti-Myc antibody and analyzed GRP78 expression by western blotting. Myc-ObRb and GRP78 constructs increase their expression levels (Fig. 21). Myc-ObRb was efficiently immunoprecipitated (Fig. 21). Within this condition, GRP78 expression level was observed in immunoprecipitates of Myc-ObRb (Fig. 21). These results suggested that GRP78 may directly interact with the long isoform of leptin receptor ObRb, which lead to modulation of leptin-induced signaling.

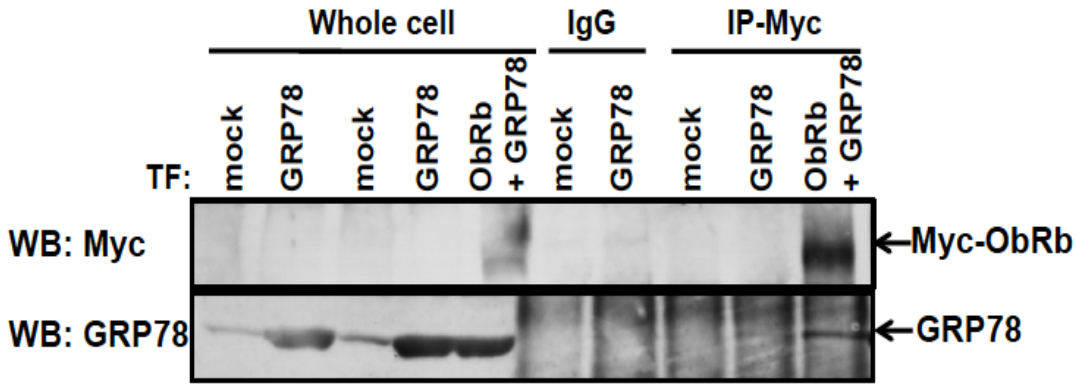


Figure 21. Interaction between GRP78 and long isoform of leptin receptor ObRb.

HEK293T cells were transfected with GRP78 and Myc-ObRb constructs. Seventy-two hours after the transfection, total cell lysates were immunopecipitated with anti-Myc tag antibody and analyzed by western bloting with the indicated antibodies. Myc was efficiently immunoprecipitated. GRP78 level was analyzed by western blotting. GRP78 directly binds to ObRb leptin receptor. Typical data of 4 independent experiments were shown.

4.4. Discussion

To date, studies on the interaction between adipocyte-derived hormone, leptin, and pancreatic hormone insulin, have been more focused on energy homeostasis (Carvalheira *et al*., 2005). Insulin was previously shown to potentiate leptin-induced STAT3 phosphorylation (Carvalheira *et al*., 2001). Furthermore, it was reported to induce GRP78 and protect against cell death (Inageda, 2010). Although a relationship between insulin and leptin has already been reported (Carvalheira *et al*., 2005; Nazarians-Armavil *et a*., 2013), the function of GRP78 in leptin signaling has not yet been revealed. In the present study, we investigated the function of GRP78 on leptin-

induced signaling in insulin-treated neuronal cells, and found that GRP78 plays an important role in the leptin-induced phosphorylation of STAT3.

Leptin and insulin, centrally regulate nutrient homeostasis by suppressing feeding desire (Woods *et* al., 1979; Ahima *et al*., 1996). Furthermore, the central administration of either peptide is known to reduce appetite (Woods *et* al., 1979; Weigle *et al*., 1995). Leptin and insulin share the common PI3K molecular pathway. In addition to the JAK2-STAT3 pathway, the PI3K pathway is one of the main pathways involved in the anti-obesity effects of leptin (Morton *et al*., 2005; Roman *et al*., 2010). The intracerebroventricular administration of a PI3K inhibitor was previously reported to inhibit the effects of leptin on anorexia (Niswender *et al*., 2001). Furthermore, insulin inhibited feeding through IRS-PI3K signaling (Niswender *et al*., 2003). Another study demonstrated that insulin induces GRP78 through the PI3K-ATF4 pathway in neuronal cells (Inageda, 2010). In the present study, we found that insulin induced the expression of GRP78, while the inhibition of GRP78 expression ameliorated insulin-induced enhancements in leptin-induced STAT3 phosphorylation. Taken together, these results suggest that by inducing GRP78, insulin has the ability to enhance leptin-induced STAT3 signaling. Furthermore, we found that the knockdown of GRP78 inhibited leptin-induced STAT3 phosphorylation, and leptin-induced STAT3 phosphorylation was enhanced by its overexpression. Hence, GRP78 may play an important role in leptin-induced STAT3 phosphorylation. Leptin resistance is a hallmark for obese patients (Münzberg and Myers, 2005), while its underlying mechanism are still being illustrated in detail. A saturated fatty acid, palmitate, was shown to induce ER stress (Karaskov *et al*., 2006). Thus, excess intake of saturated fatty acids may cause ER stress and leptin resistance in obesity. Of importance, insulin contributed to

resistance against ER stress (Inageda, 2010). Therefore, insulin may induce GRP78, thereby attenuating ER stress induced-leptin resistance in obesity.

The mechanism in which GRP78 implicates modulation of leptin-induced signals has yet to be determined. Interestingly, we found the direct interaction between GRP78 and the long isoform of leptin receptor (ObRb). Thus GRP78 may bind to the long isoform of leptin receptor, leading to an enhancement in leptin induced-STAT3 signaling pathway. GRP78 is secreted extracellularly (Arap *et al*., 2004). Thus, it would be interesting to examine the function of extracellular GRP78 on leptin-induced signal by using GRP78 neutralizing antibody or recombinant GRP78. Since GRP78 is known to promote tumor proliferation in cancer (Dong *et al*., 2008), being able to develop drug that selectively target extracellular GRP78 will be novel and safe therapeutic approach for leptin resistance.

Part 5: Conclusion

In summary, we found in the present study that increased DHAA levels (Thon *et* al., 2016) and excess in SFAs caused ER stress and leptin resistance. We also found that leptin induces GRP78 and protected against ER stress-induced cell death (Thon *et* al., 2014). Moreover, we showed that insulin enhanced leptin-induced phosphorylation of STAT3 might be dependent on GRP78 (Thon *et* al., 2016). Together, we herein to demonstrate the critical function of GRP78 on leptin signaling.

Once again, ER stress contributes to both leptin and insulin resistance in obesity. Thus, interventions that alleviate ER stress, for instance by improving protein folding via increasing chaperone capacity, would offer a potential therapeutic approach for the amelioration of obesity and ER-stress-related diseases (Fig. 22). By showing the significance of GRP78 and its underlying connection to leptin and insulin our current results may be advantageous for a more understanding the regulation of energy homeostasis, which may provide useful information for therapeutic interventions for obesity.

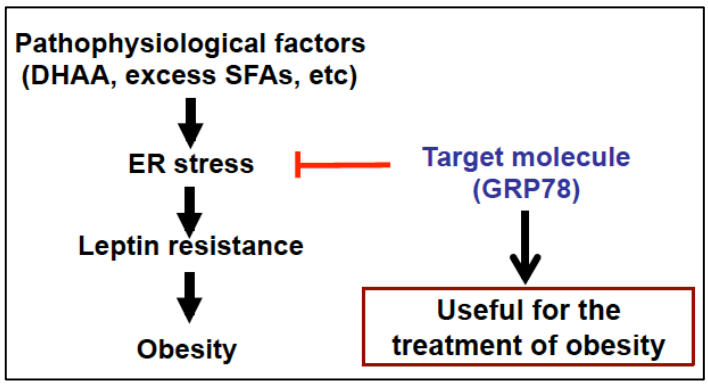


Figure 22. A schematic representation of the significant role of GRP78 in amelioration of leptin resistance.

ER stress is one of the mechanisms mediated leptin resistance. Physiological factors that induced leptin resistance also caused ER stress. Intervention that ameliorate ER stress would represent critical approach for the treatment of leptin resistance related to obesity.

References

Anelli T, Sitia R. Protein quality control in the early secretory pathway. EMBO J. 2008 Jan 23;27(2):315-27.

Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, Flier JS. Role of leptin in the neuroendocrine response to fasting. Nature. 1996 Jul 18;382(6588):250-2.

Arap MA, Lahdenranta J, Mintz PJ, Hajitou A, Sarkis AS, Arap W, Pasqualini R. Cell surface expression of the stress response chaperone GRP78 enables tumor targeting by circulating ligands. Cancer Cell. 2004 Sep;6(3):275-84.

Asilmaz E, Cohen P, Miyazaki M, Dobrzyn P, Ueki K, Fayzikhodjaeva G, Soukas AA, Kahn CR, Ntambi JM, Socci ND, Friedman JM. Site and mechanism of leptin action in a rodent form of congenital lipodystrophy. J Clin Invest. 2004 Feb;113(3):414-24.

Borradaile NM, Han X, Harp JD, Gale SE, Ory DS, Schaffer JE. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. J Lipid Res. 2006 Dec;47(12):2726-37.

Bendich A, Machlin LJ, Scandurra O, Burton GW, Wayner DDM. The antioxidant role of vitamin C, Advances in Free Radical Biology & Medicine. 2 (1986) 419-444.

Busch AK, Gurisik E, Cordery DV, Sudlow M, Denyer GS, Laybutt DR, Hughes WE, Biden TJ. Increased fatty acid desaturation and enhanced expression of stearoyl coenzyme A desaturase protects pancreatic beta-cells from lipoapoptosis. Diabetes. 2005 Oct;54(10):2917-24.

Brüning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Müller-Wieland D, Kahn CR. Role of brain insulin receptor in control of body weight and reproduction. Science. 2000 Sep 22;289(5487):2122-5.

Cui H, Cai F, Belsham DD. Leptin signaling in neurotensin neurons involves STAT, MAP kinases ERK1/2, and p38 through c-Fos and ATF1. *FASEB J* (2006) 20(14):2654-6. doi:10.1096/fj.06-5989fje

Czech MP, Corvera S. Signaling mechanisms that regulate glucose transport. J Biol Chem. 1999 Jan 22;274(4):1865-8.

Carvalheira JB, Torsoni MA, Ueno M, Amaral ME, Araújo EP, Velloso LA, Gontijo JA, Saad MJ. Cross-talk between the insulin and leptin signaling systems in rat hypothalamus. Obes Res. 2005 Jan;13(1):48-57.

Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature. 2002 Jan 3;415(6867):92-6.

Cnop M, Foufelle F, Velloso LA. Endoplasmic reticulum stress, obesity and diabetes. Trends Mol Med. 2012 Jan;18(1):59-68. doi: 10.1016/j.molmed.2011.07.010.

Carvalheira JB, Siloto RM, Ignacchitti I, Brenelli SL, Carvalho CR, Leite A, Velloso LA, Gontijo JA, Saad MJ. Insulin modulates leptin-induced STAT3 activation in rat hypothalamus. FEBS Lett. 2001 Jul 6;500(3):119-24.

Dong D, Ni M, Li J, Xiong S, Ye W, Virrey JJ, Mao C, Ye R, Wang M, Pen L, Dubeau L, Groshen S, Hofman FM, Lee AS. Critical role of the stress chaperone GRP78/BiP in tumor proliferation, survival, and tumor angiogenesis in transgene-induced mammary tumor development. Cancer Res. 2008 Jan 15;68(2):498-505.

Eizirik DL, Cardozo AK, Cnop M. The role for endoplasmic reticulum stress in diabetes mellitus. Endocr Rev. 2008 Feb;29(1):42-61.

Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet. 2006 Aug;7(8):606-19.

Fei H, Okano HJ, Li C, Lee GH, Zhao C, Darnell R, Friedman JM. Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. Proc Natl Acad Sci U S A. 1997 Jun 24;94(13):7001-5.

Friedlander R, Jarosch E, Urban J, Volkwein C, Sommer T. A regulatory link between ER-associated protein degradation and the unfolded-protein response. Nat Cell Biol. 2000 Jul;2(7):379-84.

Frei B, England L, Ames BN. Ascorbate is an outstanding antioxidant in human blood plasma. Proc Natl Acad Sci U S A. 1989 Aug;86(16):6377-81.

Flowers MT, Keller MP, Choi Y, Lan H, Kendziorski C, Ntambi JM, Attie AD. Liver gene expression analysis reveals endoplasmic reticulum stress and metabolic dysfunction in SCD1-deficient mice fed a very low-fat diet. Physiol Genomics. 2008 May 13;33(3):361-72.

Flowers MT, Ntambi JM. Role of stearoyl-coenzyme A desaturase in regulating lipid metabolism. Curr Opin Lipidol. 2008 Jun;19(3):248-56

Gautron L, Elmquist JK. Sixteen years and counting: an update on leptin in energy balance. J Clin Invest. 2011 Jun;121(6):2087-93. doi: 10.1172/JCI45888.

García-Krauss A, Ferrada L, Astuya A, Salazar K, Cisternas P, Martínez F, Ramírez E, Nualart F. Dehydroascorbic Acid Promotes Cell Death in Neurons Under Oxidative Stress: a Protective Role for Astrocytes. Mol Neurobiol. 2015 Oct 26.

Gidalevitz T, Stevens F, Argon Y. Orchestration of secretory protein folding by ER chaperones. Biochim Biophys Acta. 2013 Nov;1833(11):2410-24.

Hall KD, Heymsfield SB, Kemnitz JW, Klein S, Schoeller DA, Speakman JR. Energy balance and its components: implications for body weight regulation. Am J Clin Nutr. 2012 Apr;95(4):989-94.

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. Weight-reducing effects of the plasma protein encoded by the obese gene. Science. 1995 Jul 28;269(5223):543-6.

Helenius A, Marquardt T, Braakman I. The endoplasmic reticulum as a protein-folding compartment. Trends Cell Biol. 1992 Aug;2(8):227-31.

Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature. 1999 Jan 21;397(6716):271-4.

Hosoi T, Matsunami N, Nagahama T, Okuma Y, Ozawa K, Takizawa T, Nomura Y. 2-Aminopurine inhibits leptin receptor signal transduction. Eur J Pharmacol. 2006 Dec 28;553(1-3):61-6.

Hosoi T, Sasaki M, Miyahara T, Hashimoto C, Matsuo S, Yoshii M, Ozawa K. Endoplasmic reticulum stress induces leptin resistance. Mol Pharmacol. 2008 Dec;74(6):1610-9.

Harding HP, Ron D. Endoplasmic reticulum stress and the development of diabetes: a review. Diabetes. 2002 Dec;51 Suppl 3:S455-61.

Holtz WA, Turetzky JM, Jong YJ, O'Malley KL. Oxidative stress-triggered unfolded protein response is upstream of intrinsic cell death evoked by parkinsonian mimetics. J Neurochem. 2006 Oct;99(1):54-69.

Inageda K. Insulin modulates induction of glucose-regulated protein 78 during endoplasmic reticulum stress via augmentation of ATF4 expression in human neuroblastoma cells. FEBS Lett. 2010 Aug 20;584(16):3649-54.

Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell. 2001 Jun 29;105(7):891-902.

Jiao P, Ma J, Feng B, Zhang H, Diehl JA, Chin YE, Yan W, Xu H. FFA-induced adipocyte inflammation and insulin resistance: involvement of ER stress and IKKβ pathways. Obesity (Silver Spring). 2011 Mar;19(3):483-91.

Jiang G, Li Z, Liu F, Ellsworth K, Dallas-Yang Q, Wu M, Ronan J, Esau C, Murphy C, Szalkowski D, Bergeron R, Doebber T, Zhang BB. Prevention of obesity in mice by antisense oligonucleotide inhibitors of stearoyl-CoA desaturase-1. J Clin Invest. 2005 Apr;115(4):1030-8.

Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev. 1999 May 15;13(10):1211-33.

KC S, Cárcamo JM, Golde DW. Vitamin C enters mitochondria via facilitative glucose transporter 1 (Glut1) and confers mitochondrial protection against oxidative injury. FASEB J. 2005 Oct;19(12):1657-67.

Kelly T, Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. Int J Obes (Lond). 2008 Sep;32(9):1431-7

Kleinridders A, Schenten D, Könner AC, Belgardt BF, Mauer J, Okamura T, Wunderlich FT, Medzhitov R, Brüning JC. MyD88 signaling in the CNS is required for development of fatty acid-induced leptin resistance and diet-induced obesity. Cell Metab. 2009 Oct;10(4):249-59.

Karaskov E, Scott C, Zhang L, Teodoro T, Ravazzola M, Volchuk A. Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis. Endocrinology. 2006 Jul;147(7):3398-407.

Kawasaki N, Asada R, Saito A, Kanemoto S, Imaizumi K. Obesity-induced endoplasmic reticulum stress causes chronic inflammation in adipose tissue. Sci Rep. 2012;2:799.

Lin S, Thomas TC, Storlien LH, Huang XF. Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. Int J Obes Relat Metab Disord. 2000 May;24(5):639-46.

Lee AS. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods. 2005 Apr;35(4):373-81.

Lu J, Wang Q, Huang L, Dong H, Lin L, Lin N, Zheng F, Tan J. Palmitate causes endoplasmic reticulum stress and apoptosis in human mesenchymal stem cells: prevention by AMPK activator. Endocrinology. 2012 Nov;153(11):5275-84.

Münzberg H, Myers MG Jr. Molecular and anatomical determinants of central leptin resistance. Nat Neurosci. 2005 May;8(5):566-70.

Malnick SD, Knobler H. The medical complications of obesity. QJM. 2006 Sep;99(9):565-79.

Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. Nature. 2006 Sep 21;443(7109):289-95.

Maytin EV, Ubeda M, Lin JC, Habener JF. Stress-inducible transcription factor CHOP/gadd153 induces apoptosis in mammalian cells via p38 kinase-dependent and -independent mechanisms. Exp Cell Res. 2001 Jul 15;267(2):193-204.

Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell. 2000 May 26;101(5):451-4.

Morton GJ, Gelling RW, Niswender KD, Morrison CD, Rhodes CJ, Schwartz MW. Leptin regulates insulin sensitivity via phosphatidylinositol-3-OH kinase signaling in mediobasal hypothalamic neurons. Cell Metab. 2005 Dec;2(6):411-20.

Mayer CM, Belsham DD. Palmitate attenuates insulin signaling and induces endoplasmic reticulum stress and apoptosis in hypothalamic neurons: rescue of resistance and apoptosis through adenosine 5' monophosphate-activated protein kinase activation. Endocrinology. 2010 Feb;151(2):576-85.

May JM, Qu Z, Morrow JD. Mechanisms of ascorbic acid recycling in human erythrocytes. Biochim Biophys Acta. 2001 Oct 3;1528(2-3):159-66.

Maya-Monteiro CM, Almeida PE, D'Avila H, Martins AS, Rezende AP, Castro-Faria-Neto H, Bozza PT. Leptin induces macrophage lipid body formation by a phosphatidylinositol 3-kinase- and mammalian target of rapamycin-dependent mechanism. J Biol Chem. 2008 Jan 25;283(4):2203-10.

Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature. 2000 Jan 6;403(6765):98-103.

Ngkeekwong FC, Ng LL. Two distinct uptake mechanisms for ascorbate and dehydroascorbate in human lymphoblasts and their interaction with glucose. Biochem J. 1997 May 15;324 (Pt 1):225-30.

Ntambi JM, Miyazaki M, Stoehr JP, Lan H, Kendziorski CM, Yandell BS, Song Y, Cohen P, Friedman JM, Attie AD. Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11482-6.

Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers MG Jr, Schwartz MW. Intracellular signalling. Key enzyme in leptin-induced anorexia. Nature (2001) 413(6858):794-5.

Niswender KD, Morrison CD, Clegg DJ, Olson R, Baskin DG, Myers MG Jr, Seeley RJ, Schwartz MW. Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. Diabetes. 2003 Feb;52(2):227-31.

Ni M, Lee AS. ER chaperones in mammalian development and human diseases. FEBS Lett. 2007 Jul 31;581(19):3641-51.

Nazarians-Armavil A, Menchella JA, Belsham DD. Cellular insulin resistance disrupts leptin-mediated control of neuronal signaling and transcription. Mol Endocrinol. 2013 Jun;27(6):990-1003.

Ozcan L, Ergin AS, Lu A, Chung J, Sarkar S, Nie D, Myers MG Jr, Ozcan U. Endoplasmic reticulum stress plays a central role in development of leptin resistance. Cell Metab. 9(2009), pp. 35-51.

Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Görgün C, Glimcher LH, Hotamisligil GS. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science. 2004 Oct 15;306(5695):457-61.

Patterson JW. The diabetogenic effect of dehydroascorbic acid. Endocrinology. 1949 Sep;45(3):344.

Pfaffenbach KT, Pong M, Morgan TE, Wang H, Ott K, Zhou B, Longo VD, Lee AS. GRP78/BiP is a novel downstream target of IGF-1 receptor mediated signaling. J Cell Physiol. 2012 Dec;227(12):3803-11.

Prieur X, Roszer T, Ricote M. Lipotoxicity in macrophages: evidence from diseases associated with the metabolic syndrome. Biochim Biophys Acta. 2010 Mar;1801(3):327-37.

Pinnamaneni SK, Southgate RJ, Febbraio MA, Watt MJ. Stearoyl CoA desaturase 1 is elevated in obesity but protects against fatty acid-induced skeletal muscle insulin resistance in vitro. Diabetologia. 2006 Dec;49(12):3027-37.

Popeijus HE, Saris WH, Mensink RP. Role of stearoyl-CoA desaturases in obesity and the metabolic syndrome. Int J Obes (Lond). 2008 Jul;32(7):1076-82.

Peter A, Weigert C, Staiger H, Rittig K, Cegan A, Lutz P, Machicao F, Häring HU, Schleicher E. Induction of stearoyl-CoA desaturase protects human arterial endothelial cells against lipotoxicity. Am J Physiol Endocrinol Metab. 2008 Aug;295(2):E339-49.

Rumsey SC, Kwon O, Xu GW, Burant CF, Simpson I, Levine M. Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. J Biol Chem. 272(1997), pp. 18982-9.

Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol. 2007 Jul;8(7):519-29.

Rao RV, Peel A, Logvinova A, del Rio G, Hermel E, Yokota T, Goldsmith PC, Ellerby LM, Ellerby HM, Bredesen DE. Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78. FEBS Lett. 2002 Mar 13;514(2-3):122-8.

Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, Shulman GI. Mechanism of free fatty acid-induced insulin resistance in humans. J Clin Invest. 1996 Jun 15;97(12):2859-65.

Ratan RR, Baraban JM. Apoptotic death in an in vitro model of neuronal oxidative stress. Clin Exp Pharmacol Physiol. 1995 Apr;22(4):309-10.

Roman EA, Reis D, Romanatto T, Maimoni D, Ferreira EA, Santos GA, Torsoni AS, Velloso LA, Torsoni MA. Central leptin action improves skeletal muscle AKT, AMPK, and PGC1 alpha activation by hypothalamic PI3K-dependent mechanism. Mol Cell Endocrinol. 2010 Jan 15;314(1):62-9.

Sinclair AJ, Girling AJ, Gray L, Le Guen C, Lunec J, Barnett AH. Disturbed handling of ascorbic acid in diabetic patients with and without microangiopathy during high dose ascorbate supplementation. Diabetologia. 1991 Mar;34(3):171-5.

Som S, Basu S, Mukherjee D, Deb S, Choudhury PR, Mukherjee S, Chatterjee SN, Chatterjee IB. Ascorbic acid metabolism in diabetes mellitus. Metabolism. 1981 Jun;30(6):572-7.

Siushansian R, Tao L, Dixon SJ, Wilson JX. Cerebral astrocytes transport ascorbic acid and dehydroascorbic acid through distinct mechanisms regulated by cyclic AMP. J Neurochem. 1997 Jun;68(6):2378-85.

Sai X, Kawamura Y, Kokame K, Yamaguchi H, Shiraishi H, Suzuki R, Suzuki T, Kawaichi M, Miyata T, Kitamura T, De Strooper B, Yanagisawa K, Komano H. Endoplasmic reticulum stress-inducible protein, Herp, enhances presenilin-mediated generation of amyloid beta-protein. J Biol Chem. 2002 Apr 12;277(15):12915-20.

Takahashi Y, Okimura Y, Mizuno I, Iida K, Takahashi T, Kaji H, *et al*. Leptin induces mitogen-activated protein kinase-dependent proliferation of C3H10T1/2 cells. *J Biol Chem* (1997) 272(20):12897-900. doi:10.1074/jbc.272.20.12897

Takano A, Usui I, Haruta T, Kawahara J, Uno T, Iwata M, Kobayashi M. Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. Mol Cell Biol. 2001 Aug;21(15):5050-62.

Thon M, Hosoi T, Ozawa K. Dehydroascorbic acid-induced endoplasmic reticulum stress and leptin resistance in neuronal cells. Biochem Biophys Res Commun. 2016 Sep 16;478(2):716-20.

Thon M, Hosoi T, Yoshii M, Ozawa K. Leptin induced GRP78 expression through the PI3K-mTOR pathway in neuronal cells. Sci Rep. 2014 Nov 18;4:7096. doi: 10.1038/srep07096.

Thon M, Hosoi T, Ozawa K. Insulin enhanced leptin-induced STAT3 signaling by inducing GRP78. Sci Rep. 2016 Sep 28;6:34312.

Vera JC, Rivas CI, Fischbarg J, Golde DW. Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. Nature. 1993 Jul 1;364(6432):79-82.

Vaisse C, Halaas JL, Horvath CM, Darnell JE Jr, Stoffel M, Friedman JM. Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. Nat Genet. 1996 Sep;14(1):95-7.

Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, Boorstein R, Kreibich G, Hendershot LM, Ron D. Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). Mol Cell Biol. 1996 Aug;16(8):4273-80.

Won JC, Jang PG, Namkoong C, Koh EH, Kim SK, Park JY, Lee KU, Kim MS. Central administration of an endoplasmic reticulum stress inducer inhibits the anorexigenic effects of leptin and insulin. Obesity (Silver Spring). 17(2009):1861-5.

Werstuck GH, Lentz SR, Dayal S, Hossain GS, Sood SK, Shi YY, Zhou J, Maeda N, Krisans SK, Malinow MR, Austin RC. Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. J Clin Invest. 2001 May;107(10):1263-73.

Wennström S, Downward J. Role of phosphoinositide 3-kinase in activation of ras and mitogen-activated protein kinase by epidermal growth factor. Mol Cell Biol. 1999 Jun;19(6):4279-88.

Woods SC, Lotter EC, McKay LD, Porte D Jr. Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. Nature. 1979 Nov 29;282(5738):503-5.

Weigert C, Brodbeck K, Staiger H, Kausch C, Machicao F, Häring HU, Schleicher ED. Palmitate, but not unsaturated fatty acids, induces the expression of interleukin-6 in human myotubes through proteasome-dependent activation of nuclear factor-kappaB. J Biol Chem. 2004 Jun 4;279(23):23942-52.

Weigle DS, Bukowski TR, Foster DC, Holderman S, Kramer JM, Lasser G, Lofton-Day CE, Prunkard DE, Raymond C, Kuijper JL. Recombinant ob protein reduces feeding and body weight in the ob/ob mouse. J Clin Invest. 1995 Oct;96(4):2065-70.

Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell. 2001 Dec 28;107(7):881-91.

Yin J, Wang Y, Gu L, Fan N, Ma Y, Peng Y. Palmitate induces endoplasmic reticulum stress and autophagy in mature adipocytes: implications for apoptosis and inflammation. Int J Mol Med. 2015 Apr;35(4):932-40

Zhang X, Zhang G, Zhang H, Karin M, Bai H, Cai D. Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. Cell. 2008 Oct 3;135(1):61-73.

Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev. 12(1998), pp. 982-95.

Zheng M, Zhang Q, Joe Y, Kim SK, Uddin MJ, Rhew H, Kim T, Ryter SW, Chung HT. Carbon monoxide-releasing molecules reverse leptin resistance induced by endoplasmic reticulum stress. Am J Physiol Endocrinol Metab. 2013 Apr 1;304(7):E780-8.

Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med. 1995 Aug 1;182(2):367-77.

List of Reagents

Human leptin	(Enzo Life Science, SE161-0001)
(L)-Dehydroascorbic acid	(Sigma-Aldrich, 261556)
Palmitate	(Tokyo Chemical Industry, P0007)
BSA	(Sigma, A6003)
Rapamycin	(Santa Cruz, SC-3504)
Tunicamycin (Tm)	(Wako Pure Chemical Ltd., 202-08241)
Thapsigargin (Tg)	(Wako Pure Chemical Ltd., 205-17283)
LY294002	(Sigma, L9908)
PD98059	(RBI, P215)
Human insulin (Humulin®R)	(Eli Lilly and Company, 0002-8215)
SCD1 inhibitor (CAY10566)	(Cayman Chemical, 10012562)
anti-phospho-STAT3 (Tyr705) antibody	(Cell Signaling, #9145)
anti-STAT3 antibody	(Cell Signaling, # 9132)
anti-SCD1 antibody	(Cell Signaling, #2438)
anti-GAPDH antibody	(Acris, ACR001P)
anti-phospho-Akt (Thr308) antibody	(Cell Signaling, #9275)
rabbit anti-phospho-ERK antibody (Thr202/Tyr204)	(Cell Signaling, #39191)
anti-CHOP antibody	(Santa Cruz, G1708)
anti-phospho-IRE1α (phospho S724) antibody	(Abcam, ab48187)
anti-KDEL antibody	(Enzo Life Sciences; #02231116)
anti-phospho-S6K (Thr389) antibody	(Cell Signaling, #9234)
anti-ERK1/2 antibody	(StressMarq, SPC-120C)
anti-AKT antibody	(Cell signaling, #9272)
anti-STAT3 antibody	(Cell signaling, # 9132)

anti-IRE1α antibody	(Cell signaling, #3294)
anti-S6K antibody	(Cell signaling, #2708)
anti-GRP78 (GL-19) antibody	(Sigma-Aldrich, G8918)
anti-phospho-S6K (Thr389) antibody	(Cell signaling, #9234)
anti-GAPDH antibody	(Millipore, #MAB374)
anti-Myc-tag mAb antibody	(M192-3, MBL)

Acknowledgement

Foremost, I would like to express my sincere gratitude to my advisor, professor Koichiro Ozawa for his commitment to accept me to be a part in his department and to develop my skills in research. Without his guidance, I will not be able to reach my dream as a researcher. He is a unique advisor and understands clearly how to approach his professionalism setting and passion. I cannot imagine a better advisor I meet.

I deeply grateful to Dr. Toru Hosoi, associate professor of Department of Pharmacotherapy, Hiroshima University, who always makes many valuable suggestions and gives me constitutive advice. He has been a key asset in my study and my laboratory experience.

I also would like to thank Dr. Michiko Yoshii, assistant professor of Department of Pharmacotherapy, Hiroshima University, for her instruction in basic technique of research.

I am also grateful to thank Professor Takashi Takata, professor of Department of Oral and Maxillofacial Pathobiology, Hiroshima University, who gives me a chance to observe the basis of laboratory works in his department.

My sincere thank to Dr. Kaoru Hazeki to be a co-advisor of my research project.

It is pleasure to express my gratitude to Professor Osamu Hazeki, professor Norimitsu Morioka, and Dr. Yaichiro Kotake, the members of my PhD committee, who monitor my work and provide me with valuable comments on my thesis.

I would like to express by profound thank to Kobayashi International Scholarship Foundation that provides financial supports for my research.

I would like to thank all members of Department of Pharmacotherapy, Hiroshima University, for providing an inspiring working atmosphere.

I would like to acknowledge MEXT (Ministry of Education, Culture, Sports, Science, and Technology of Japan) for 2 years financial support of my PhD course.

I must also show my wholehearted acknowledge my husband and daughters for their assistance and encouragement.