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Endoplasmic reticulum stress induces leptin resistance

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Running title: ER stress induces leptin resistance

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Abbreviations: ER stress, endoplasmic reticulum stress; STAT3, signal transducer and activator of transcription 3; JAK2, janus kinase 2; JNK, jun amino terminal kinase; PTP1B, protein tyrosine phosphatase 1B; SOCS3, suppressors of cytokine signaling 3; UPR, unfolded protein response; GRP78, glucose regulated protein 78; CHOP, CCAAT/enhancer-binding protein homologous protein

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Abstract

Leptin is an important circulating signal for inhibiting food intake and body weight gain. In recent years, “leptin resistance” has been considered to be one of the main causes of obesity. However, the detailed mechanisms of leptin resistance are poorly understood. Increasing evidence has suggested that stress signals, which impair endoplasmic reticulum (ER) function lead to an accumulation of unfolded proteins, which results in ER stress. In the present study, we hypothesized that ER stress is involved in leptin resistance. Tunicamycin, thapsigargin, or brefeldin A were used to induce ER stress. The activation status of leptin signals was measured by Western blotting analysis using a phospho (Tyr705)-signal transducer and activator of transcription 3 (STAT3) antibody. We observed that ER stress markedly inhibited leptin-induced STAT3 phosphorylation. In contrast, ER stress did not affect leptin-induced jun amino terminal kinase (JNK) activation. These results suggest that ER stress induces leptin resistance. ER stress-induced leptin resistance was mediated through protein tyrosine phosphatase 1B (PTP1B) but not through suppressors of cytokine signaling 3 (SOCS3). Importantly, a chemical chaperone, which could improve the protein-folding capacity, reversed ER stress-induced leptin resistance. Moreover, homocysteine, which induces ER stress, caused leptin resistance both *in vitro* and *in vivo*. Together, these findings suggest that the pathologic mechanism of leptin resistance is derived from ER stress.

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Introduction

Leptin is an important circulating signal for inhibiting food intake and body weight gain through its actions in the brain (Campfield et al., 1995; Zhang et al., 1994). The leptin receptor (Ob-Rb) has a consensus amino acid sequence involved in the activation of JAK-STAT tyrosine kinases, and activates the signal transducer and activator of transcription 3 (STAT3) protein in the hypothalamus and brain stem (Hosoi et al., 2002a; Vaisse et al., 1996). Leptin activates janus kinase 2 (JAK2), which in turn phosphorylates residues of Tyr985 and Tyr1138 within the Ob-Rb leptin receptor (Li et al., 1999). Phosphorylated Tyr985 results in leptin-induced extracellular signal related kinase (ERK) activation (Banks et al., 2000). On the other hand, phosphorylated Tyr1138 results in leptin-induced STAT3 activation (Banks et al., 2000). The essential role of the Ob-Rb-STAT3 signal was demonstrated by the finding that the replacement of Tyr 1138 in Ob-Rb with a serine residue (S1138), which specifically disrupts the Ob-Rb-STAT3 signal, results in marked obesity (Bates et al., 2003).

As leptin has an anti-obesity action, it was initially expected that exogenous leptin therapy might be effective in treating obesity. However, most obese individuals have elevated circulating levels of leptin and they do not adequately respond to these increased leptin levels. Such unresponsiveness to elevated leptin levels gave rise to the idea that obesity is associated with “leptin resistance”. In recent years, leptin resistance has been considered to be one of the main causes of the pathologic development of obesity (Munzberg et al., 2005). Thus, elucidation of the mechanism

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of leptin resistance is an important subject in respect to treating obesity. It has been suggested that suppressors of cytokine signaling 3 (SOCS3) (Bjørbæk et al., 1998; Bjørbæk et ak., 1999) or protein tyrosine phosphatase 1B (PTP1B) (Cheng et al., 2002; Zabolotny et al., 2002) are involved in leptin resistance. However, the detailed mechanisms of leptin resistance are poorly understood.

The endoplasmic reticulum (ER) is an important organelle that is responsible for the correct folding and sorting of proteins. Stress signals, which impair ER function, would lead to an accumulation of unfolded proteins. Recent studies indicate that moderate stress on ER will trigger many rescuer responses, including an unfolded protein response (UPR). The UPR acts to alleviate ER stress by: 1) increasing the folding capacity, 2) inhibiting general protein translation, and 3) promoting the degradation of misfolded proteins (Mori, 2000; Yoshida et al., 2001). On the other hand, excessive or long-term exposure to ER stress induces apoptosis (Hyoda et al., 2006; Nakagawa et al., 2000; Zinszner et al., 1998). Increasing evidence suggests that ER stress is implicated in the pathogenesis of diseases such as Alzheimer's and Parkinson's disease (Nakagawa et al., 2000; Imai et al., 2001; Katayama., 1999; Xu et al., 2005). However, the possible involvement of ER stress in obesity (leptin resistance) is not understood. Thus, in the present study, we hypothesized that ER stress is involved in leptin resistance.

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Results

ER stress induced leptin resistance

ER stress is induced by agents that interfere with protein glycosylation (i.e., tunicamycin), Ca^{2+} balance (i.e., thapsigargin) or ER-Golgi vesicular transport (i.e., brefeldin A). The conditions of ER stress trigger the cellular UPR, which results in an increase in UPR-regulated genes such as glucose regulated protein 78 (GRP78), an ER-resident chaperone, or CCAAT/enhancer-binding protein homologous protein (CHOP), an ER stress-induced apoptotic transcription factor. We observed that ER stress reagents (tunicamycin, thapsigargin, and brefeldin A) induced GRP78 and CHOP in HEK293 and SH-SY5Y neuroblastoma cell lines stably transfected with the Ob-Rb leptin receptor (HEK293-Ob-Rb and SH-SY5Y-Ob-Rb, respectively), as assessed by RT-PCR and Western blotting (Fig. 1AB). The results suggest that ER stress was induced. On the other hand, ER stress did not alter expression level of leptin receptor (Fig. 1C). Thus, we next investigated whether ER stress alters the leptin signal. Treatment with leptin (0.5 μg/mL, 15 min) increased STAT3 phosphorylation in the HEK293 Ob-Rb or SH-SY5Y Ob-Rb cell line, indicating that the leptin signal was activated under the present experimental conditions (Fig. 2AB&E-H). As shown in Fig. 2AB, leptin-induced STAT3 phosphorylation was clearly inhibited in ER-stressed (tunicamycin- or thapsigargin-treated) HEK293-Ob-Rb cells. Low-dose leptin (0.03 μg/mL)-induced STAT3 phosphorylation was also inhibited by ER stress (data not shown). Another ER stress inducer, brefeldin A, also inhibited leptin-induced STAT3 phosphorylation in HEK293-Ob-Rb cells (data not shown). Similar results were

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obtained in the SH-SY5Y-Ob-Rb neuroblastoma cell line (Fig. 2I-L). Leptin-induced ERK phosporylation was also inhibited by ER stress-inducing reagent (Fig. 2CD). The inhibitory effects of ER stress on leptin-induced STAT3 phosphorylation were time (0.5-4 h)- and dose (Tm: 0.01-10 μg/mL, Tg: 0.001-1 μM)-dependent (Fig. 3). On the other hand, we did not observe any change in cell viability by these stressors at 4 h time point (data not shown). Thus, through investigating STAT3 phosphorylation as an indicator of the leptin signal, the present results suggest that ER stress induces leptin resistance.

As we observed that ER stress inhibited leptin-induced STAT3 phosphorylation, we subsequently investigated whether ER stress would affect the phosphorylation status of global proteins. As shown in Fig. 2M, cellular tyrosine phosphorylation in ER-stressed cells was similar to that in control cells, indicating that ER stress does not perturb global tyrosine kinase pathways (Fig. 2M). The results suggest that ER stress specifically inhibit leptin-induced STAT3 signal transduction. Leptin has been reported to induce the NH2-terminal c-Jun kinase/stress-activated protein kinase pathway (Bouloumie et al., 1999). Therefore, we investigated the effect of ER stress on leptin-induced c-Jun N-terminal kinase (JNK) phosphorylation. Leptin alone increased JNK phosphorylation (Fig. 2EF). In consistent with previous report (Urano et al., 2000), we observed an increase in JNK phosphorylation in ER stressed cells, and leptin-induced JNK phosphorylation was not inhibited by ER stress (Fig. 2E-H).

Chemical chaperones reversed ER stress-induced leptin resistance

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Chemical chaperones such as 4-phenyl butyric acid (4-PBA) can stabilize the protein conformation and improve the folding capacity of ER (de Almeida et al., 2007; Welch et al., 1996). Thus, we subsequently investigated the effect of 4-PBA on ER stress-induced leptin resistance. As shown in Fig. 4, 4-PBA significantly reversed the inhibitory effect of ER stress on leptin-induced STAT3 phosphorylation. These results suggest that ER stress is involved in leptin resistance, and that the abrogation of ER stress by 4-PBA could improve leptin resistance.

ER stress-induced leptin resistance is mediated independently through SOCS3

We attempted to clarify the mechanisms of the inhibitory effects of ER stress on leptin signals. SOCS3 is the feedback regulator of JAK-STAT pathways responsible for switching off cytokine signals (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). SOCS3 has been reported to be induced by leptin in the hypothalamus, and SOCS3 expression can block leptin-induced signal transduction (Bjørbæk et al., 1998; Bjørbæk et al., 1999). These observations indicate that SOCS3 is a leptin-induced negative feedback regulator. Thus, we next investigated the possible involvement of SOCS3 on ER stress-induced leptin resistance. Consistent with previous reports (Bjørbæk et al., 1998; Bjørbæk et al., 1999), we observed an increase in SOCS3 expression in leptin-treated (0.5 μg/mL, 6 h) SH-SY5Y-Ob-Rb cells (Fig. 5E-H). Therefore, we investigated whether ER stress would affect SOCS3 levels. As shown in Fig. 5, ER stress-inducing reagents increased GRP78 expression, indicating the induction of ER stress in the present conditions (Fig. 5A-D). However, ER stress did

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not induce SOCS3 at the times investigated (Fig. 5E-H). Moreover, the expression level of SOCS3 was not affected in the same samples, which inhibited leptin-induced STAT3 phosphorylation in ER-stressed cells (Fig. 5I-L). Together, these findings suggest that SOCS3 may not be involved in ER stress-induced leptin resistance.

ER stress-induced leptin resistance is mediated through protein tyrosine phosphatase 1B (PTP1B)

In addition to SOCS3, recent evidence has suggested that PTP1B is involved in leptin resistance (Cheng et al., 2002; Zabolotny et al., 2002; Bence et al., 2006). We therefore decided to investigate the possible involvement of PTP1B in ER stress-induced leptin resistance using 3-(3,5-Dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-yl sulfamyl)-phenyl)-amide, a selective, reversible, and non-competitive allosteric inhibitor of PTP1B (Wiesmann et al., 2004). The allosteric site in PTP1B binds small molecules and is not well-conserved among phosphatases, leading to the selectivity of this inhibitor (Wiesmann et al., 2004). HEK293-Ob-Rb cells were treated with Tm (1 μg/mL) or Tg (0.1 μM) for 4 h and we analyzed PTP1B activity using DuoSet IC (R&D Systems, USA). Tm or Tg increased PTP1B activity, and this effect was inhibited on treatment with the with PTP1B inhibitor (data not shown). Consistent with previous reports using $PTP1B^{-/-}$ fibroblasts (Zabolotny et al., 2002), total cellular tyrosine phosphorylation in PTB1B inhibitor-treated cells was similar to that in control cells (Fig 6A), indicating the specificity of the PTP1B inhibitor and arguing against the global

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pertubation of tyrosine kinase pathways in the absence of PTP1B. As shown in Fig. 6BC, ER stress inhibited leptin-induced STAT3 phosphorylation and the inhibitory effects were significantly reversed on the treatment with PTP1B inhibitor.

To confirm the selectivity of the inhibitor, we performed RNAi experiments. First, the knockdown potency of PTP1B siRNA was characterized. Transfecting PTP1B siRNA inhibited the expression level of PTP1B in the SH-SY5Y-Ob-Rb cell line (Fig. 6DE). Thus, we then investigated whether knocking down PTP1B would restore ER stress-induced leptin resistance. As shown in Fig. 6FG, ER stress-induced leptin resistance was reversed by transfecting PTP1B siRNA. A similar result was observed using another sequence of PTP1B siRNA (data not shown). Together, these findings suggest that the ER stress-induced inhibition of the leptin signal is dependent on PTP1B.

Homocysteine induced leptin resistance

As we observed that ER stress induced leptin resistance, we subsequently investigated the effect of a physiological regulator of ER stress on leptin signals. Recently, homocysteine was reported to induce ER stress (Kokame et al., 1996; Outinen et al., 1999). So, we next examined the effect of homocysteine on the leptin-induced STAT3 signal.

Homocysteine induced GRP78 expression in HEK293-Ob-Rb cells (Fig. 7AB), indicating that homocysteine causes ER stress. Therefore, we investigated the effect of homocysteine on the leptin-induced activation of the STAT3 signal. As shown in

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Fig. 7, we found that homocysteine dose (1-10 mM)- and time (1-4 h)- dependently inhibited leptin-induced STAT3 phosphorylation in the HEK 293 Ob-Rb cell line (Fig. 7C-F). These results suggest that homocysteine induces leptin resistance.

To further determine the physiological consequence of homocysteine's effect on leptin resistance, we performed an *in vivo* experiment. Homocysteine (i.p.) increased ER stress in the hypothalamus, as monitored by XBP-1 splicing (unpublished observation). We injected homocysteine into mice 1 h before leptin injection, and the phosporylation status of STAT3 in the hypothalamus was analyzed by Western blotting. As shown in Fig. 8, leptin (i.v., 30 min) increased STAT3 phosphorylation in the hypothalamus, and homocysteine (i.p., 1 h) significantly inhibited leptin-induced STAT3 phosphorylation. Together, these *in vitro* and *in vivo* data suggest that homocysteine is involved in leptin resistance.

Discussion

In the present study, through investigating STAT3 phosphorylation as an indicator of the leptin signal, we have shown that ER stress is involved in leptin resistance. Moreover, we have shown that PTP1B but not SOCS3 is involved in ER stress-induced leptin resistance. 4-PBA is a chemical chaperone, which can stabilize the protein conformation and improve the protein-folding capacity of ER. We found that 4-PBA reversed ER stress-induced leptin resistance, strengthening ER stress as a mediator of leptin resistance. As homocysteine (which induces ER stress) inhibited leptin-induced STAT3 phosphorylation, homocysteine may be involved in leptin resistance. Overall, these observations led us to hypothesize that ER stress may be an important, novel factor involved in leptin resistance.

Leptin activates STAT3 protein through the Ob-Rb long isoform of the leptin receptor but not through the short isoform (Vaisse et al., 1996; Ghilardi et al., 1996; Hosoi et al., 2002b). On the other hand, leptin activates the MAPK pathway through the short and long isoforms (Bjørbæk et al., 1997). The essential role of the Ob-Rb-STAT3 signal was demonstrated by the observation that db/db mice (which lack the functional Ob-Rb long isoform of leptin receptor) caused obesity, as characterized by increased food intake and body weight. Moreover, the replacement of Tyr 1138 in Ob-Rb with a serine residue (S1138), which specifically disrupts the Ob-Rb-STAT3 signal, results in marked obesity (Bates et al., 2003). These observations indicate that the leptin-induced STAT3 signal is important in regulating (reducing) obesity. In the present study, we focused on changes in STAT3 phosphorylation as an indicator of

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leptin resistance. We found that ER stress inhibited leptin-induced STAT3 phosphorylation. Thus, it is suggested that ER stress could contribute to leptin resistance. It is interesting subject to investigate whether ER stress would affect activation status of leptin receptor it's self (such as conformation and/or phosphorylation status). On the other hand, ER stress has been shown to prolong the growth hormone-induced JAK2/STAT5 signaling pathway (Flores-Morales A et al., 2001), raising the possibility that ER stress does not impair signaling via other receptors. Therefore, in addition to the leptin signal, it would be of interest to further investigate the role of ER stress in other signals, such as cytokine and/or growth hormone-mediated signal transduction.

The mechanism of ER stress-induced leptin resistance is unknown. We therefore attempted to clarify the molecular mechanisms of ER stress-induced leptin resistance. In the present study, we investigated the possible involvement of SOCS3 on ER stress-induced leptin resistance as it has been suggested to be involved in leptin resistance (Bjørbæk et al., 1998; Bjørbæk et al., 1999). However, we did not observe an association between SOCS3 and ER stress-induced leptin resistance. These data may indicate that ER stress induces leptin resistance independently through SOCS3. On the other hand, recent studies have indicated that PTP1B is involved in leptin resistance (Cheng et al., 2002; Zabolotny et al., 2002; Bence et al., 2006). We therefore investigated the possible involvement of PTP1B on ER stress-induced leptin resistance, and found that the PTP1B inhibitor significantly reversed ER stress-induced leptin resistance. We also observed that RNAi-mediated knockdown of PTP1B

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reversed ER stress-induced leptin resistance. Overall, these results suggest that PTP1B would be involved in ER stress-induced leptin resistance. Further experiments, such as dominant negative or knock out studies for PTP1B would be necessary to strengthen our PTP1B inhibitor and RNAi results. Interestingly, PTP1B has been shown to potentiate IRE1 signaling during endoplasmic reticulum stress (Gu et al., 2004). Thus, our results clearly demonstrate that ER stress-induced leptin resistance is mediated through PTP1B, and that the PTP1B inhibitor may be effective in treating obesity. Furthermore, it would be interesting to investigate these possibilities using animal models such as PTP1B-deficient mice to support our hypothesis.

As we found that ER stress is involved in leptin resistance, we further investigated the effect of a physiological regulator of ER stress on leptin signals. Homocysteine is formed upon the demethylation of methionine, and hyperhomocysteinemia is associated with cardiovascular disease, neurodegeneration, and diabetes. However, the possible involvement of hyperhomocysteinemia in leptin resistance has not been explored. As homocysteine induces ER stress (Kokame et al., 1996; Outinen et al., 1999), we next investigated possible involvement of homocysteine on leptin resistance. In the present study, we found that homocysteine induces ER stress and causes leptin resistance. These results suggest that homocysteine may be a mediator of leptin resistance. Indeed, the plasma homocysteine level is increased in obese patients compared with non-obese controls (Narin et al., 2005). Moreover, the plasma homocysteine levels were positively associated with the serum leptin levels (Narin et al., 2005). Thus, the results of our experiments clearly show that homocysteine would be one of the factors

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involved in leptin resistance. It is possible that high levels of plasma homocysteine would result in ER stress, which may causes leptin resistance. On the other hand, homocysteine has been reported to cause not only ER stress but also affect multiple signaling pathways such as oxidant stress and inflammation (Papatheodorou et al., 2007). Thus, we cannot rule out the possibility that other mechanism(s) are also involved in homocystein-induced leptin resistance. It would be interesting to further investigate the molecular mechanism(s) of homocysteine-induced leptin resistance in future studies.

Increasing evidence suggests that ER stress is involved in the pathogenesis of diseases such as Alzheimer's and Parkinson's disease. ER stress was reported to increase in obesity (Ozcan et al., 2004). However, to our knowledge, the relation between leptin resistance and ER stress has not been reported. We found that ER stress is one of the important factors involved in leptin resistance. In addition, we observed that 4-PBA (a chemical chaperone, which enhances the protein-folding capacity) reversed ER stress-induced leptin resistance. 4-PBA has been used for the clinical treatment of urea cycle disorders in children, sickle cell disease, thalassemia, and cystic fibrosis (Perlmutter et al., 2002). The results of clinical trials have shown that 4-PBA has few side effects and is safe (Carducci et al., 2001). Thus, our results suggest that 4-PBA might be a candidate drug for treating leptin resistance and that it provides basic information for treating ER stress-related diseases.

Herein, we have described a novel mechanism of leptin resistance, i.e., involvement of ER stress. Moreover, our results identify a previously undescribed class of drug for

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treating “leptin resistance”, which targets ER stress. The further understanding of ER stress-induced leptin resistance may be critical to clarify the molecular mechanism/appropriate pharmacological treatment of obesity.

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Materials and Methods

Materials and reagents- Tunicamycin and thapsigargin were obtained from Wako Pure Chemical Industries, Ltd. (Japan). 4-phenyl butyric acid was obtained from Nacalai Tesque (Japan). DL-homocysteine and brefeldin A were purchased from SIGMA (St. Louis, MO, USA). 3-(3,5-Dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-yl sulfamyl)-phenyl)-amide (PTP1B inhibitor) was obtained from Calbiochem.

Cell culture- Human HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and antibiotics (100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B; Nacalai Tesque, JAPAN) at 37°C in humidified 5% CO_2 and 95% air. Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37°C in humidified 5% CO_2 and 95% air. All experiments were performed in Dulbecco's modified Eagle's medium containing serum and antibiotics.

Generation of Ob-Rb leptin receptor-stable transfectant cell line- The human Ob-Rb leptin receptor construct was a kind gift from Genetech Inc. The Ob-Rb leptin receptor construct was transfected into SH-SY5Y and HEK293 cells using LipofectAMINE PLUS Reagent (Life Technologies Inc.) according to the manufacturer’s instructions. After the transfection, stable transfectants were obtained

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by selection with the antibiotic G418 (Hosoi et al., 2006).

RNAi experiment- PTPN1 validated Stealth^{TM} RNAi Duo Pak siRNAs (Invitrogen) was used for PTP1B siRNA. Stealth RNAi-negative CTL LO GC (Invitrogen) was used for control siRNA. Transient transfections of siRNAs were performed in 80-90% confluent SH-SY5Y (Ob-Rb) cells. TransIT-TKO Transfection Reagent (Mirus) was used to transfect siRNA, and the transfection was performed according to the manufacturer's protocol. Final concentrations of siRNAs used in the present experiments were 25 nM.

Animals-C57BL/6 mice were maintained in a room at 22-24°C under a constant day-night rhythm and given food and water ad libitum. All animal experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Hiroshima University.

Leptin and homocysteine injection and sample preparation- Before leptin and homocysteine injections, mice were given food and water ad libitum. Homocysteine was dissolved in 0.5 N HCl, and all injections were administered intraperitoneally, dosed at 0.5 mg/g. Murine leptin (1 μg/g, Pepro Tech, London, UK) was dissolved in saline, and all injections were administered intravenously via the tail vein. Mice were sacrificed by decapitation and the brain was quickly removed. The hypothalamus was rapidly dissected out onto an ice-cold plate. Then, the samples were snap-frozen in

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liquid nitrogen and stored at -80°C. For Western blotting, tissue samples were homogenized in a 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 PMSF, and 1% NP-40. The samples were centrifuged at 20,630 g for 45 min at 4°C, and supernatants were collected. The samples were boiled with laemmli buffer for 3 min.

Western blotting analysis- Western blotting was performed as described previously (Hosoi et al., 2002a; Hosoi et al., 2006). Cells were washed with ice-cold PBS and lysed in a 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 (PMSF), and 1% NP-40 for 20 min. The lysates were centrifuged at 15,000 rpm for 20 min at 4 °C, and supernatants were collected. The samples were boiled with laemmli buffer for 3 min, fractionated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred at 4°C to nitrocellulose membranes. The membranes were incubated with anti-phospho STAT3 (Tyr705: Cell Signaling; diluted to 1:1,000), anti-STAT3 (Santa Cruz; diluted to 1:1,000), anti-Ob-R (Santa Cruz; diluted to 1:300), anti-PTP1B (Calbiochem; a diluted to 1:1,000), anti-phospho ERK (Thr202/Tyr204: Cell Signaling; diluted to 1:1,000), anti-phospho JNK (Thr183/Tyr185: Cell Signaling; diluted to 1:1,000), anti-phospho Tyr (Upstate; diluted to 1:1,000), anti-SOCS3 (Santa Cruz; diluted to 1:100), and anti-KDEL (StressGen; diluted to 1:1,000) antibodies, followed by incubation with

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anti-horseradish peroxidase-linked antibody. Peroxidase was detected by chemiluminescence using an enhanced chemiluminescence system.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis- Total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA). RT-PCR was performed as described previously (Hosoi et al., 2002a). Specifically, cDNA was synthesized from total RNA by reverse transcription using 100 U of Superscript III Reverse Transcriptase (Invitrogen) and Oligo $(dt)_{12-18}$ primer (Invitrogen) in a 20-μL reaction mixture containing Superscript buffer (Invitrogen), 1 mM dNTP mix, 10 mM dithiothreitol (DTT), and 40 U of RNase inhibitor. Total RNA and Oligo $(dt)_{12-18}$ primer were incubated at 70°C for 10 min prior to reverse transcription. After incubation for 1.5 h at 46°C, the RT reaction was terminated by denaturing the reverse transcriptase for 15 min at 70°C. For PCR amplification, 1.2 μL of cDNA was added to 12 μL of a reaction mix containing 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 (GeneAmp® PCR System 9700). The following primers were used: GRP78 upstream, 5'-tgc ttg atg tat gtc ccc tta-3'; GRP78 downstream, 5'-cct tgt ctt cag ctg tca ct-3'; CHOP upstream, 5'-gca cct ccc aga gcc ctc act ctc c-3'; CHOP downstream, 5'-gtc tac tcc aag cct tcc ccc tgc g-3'; PTP1B upstream, 5'-ctg cct ctt gct gat gga ca-3'; PTP1B downstream, 5'-acg acc cga ctt cta act tc-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) upstream, 5'-aat ccc atc acc atc ttc cag -3'; and GAPDH

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downstream, 5'-agg ggc cat cca cag tct tct-3'. The PCR products (10 μL) were resolved by electrophoresis in an 8% polyacrylamide gel in TBE buffer. The gels were stained with ethidium bromide and then photographed under ultraviolet light. cDNAs for GRP78, CHOP, PTP1B, and GAPDH were amplified for 19 (94°C 45 sec, 54°C 1 min, 72°C 45 sec), 22 (94°C 45 sec, 63°C 1 min, 72°C 45 sec), 28 (94°C 1 min, 57°C 45 sec, 72°C 45 sec), and 18 (94°C 1 min, 57°C 1 min, 72°C 1 min) cycles, respectively, and these PCR reactions were run separately. These cycle numbers were chosen based on a preliminary study determining the linear range of amplification for each respective molecule.

Statistics- Results are expressed as the means ± S.E.. Statistical analysis was performed using Student's *t*-test, paired *t*-test and the Dunnett's test.

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Figure legends

Fig. 1 ER stress induced GRP78 and CHOP expression in HEK293-Ob-Rb and SH-SY5Y-Ob-Rb cells

(A) HEK293-Ob-Rb cells or SH-SY5Y cells were treated with tunicamycin (Tm: 1 μg/mL) or thapsigargin (Tg: 0.1 μM) for 6 h and expression levels of GRP78 or CHOP were analyzed by RT-PCR. **(B)** HEK293-Ob-Rb cells were treated with tunicamycin (Tm: 1 μg/mL), thapsigargin (Tg: 1 μM), or brefeldin A (Bre: 0.1 μg/mL) for 24 h and expression levels of GRP78 were analyzed by Western blotting. SH-SY5Y-Ob-Rb cells were treated with tunicamycin (Tm: 1 μg/mL) or brefeldin A (Bre: 0.1 μg/mL) for 18 h and expression levels of GRP78 was analyzed by Western blotting. **(C)** SH-SY5Y-ObRb cells were treated with tunicamycin (Tm : 1μg/mL) or brefeldin A (Bre : 0.03μg/mL) for 4 h. Western blotting analysis was performed using specific antibodies for Ob-R and GAPDH.

Fig. 2 ER stress induced leptin resistance

(AB) HEK293-Ob-Rb cells were treated with tunicamycin (Tm: 1 μg/mL) or thapsigargin (Tg: 0.1 μM) for 4 h, and then stimulated with leptin (0.5 μg/mL) for 15 min. Phospho-STAT3 (Tyr705) and STAT3 levels were analyzed by Western blotting. **(CD)** SH-SY5Y-ObRb cells were treated with brefeldin A (Bre : 0.03 μg/mL) for 4 h and then stimulated with leptin (0.5μg/mL) for 15 min. Western blotting analysis was performed using specific antibodies for phospho-ERK (Thr202/Tyr204). **(EF)** HEK293-Ob-Rb cells were treated with tunicamycin (Tm: 1 μg/mL, 2 h) or

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thapsigargin (Tg: 0.1 μM, 0.5 h). Leptin (0.5 μg/mL) was stimulated for 15 min. The phospho-JNK level was analyzed by Western blotting. **(GH)** HEK293-Ob-Rb cells were treated with tunicamycin (Tm: 1 μg/mL) or thapsigargin (Tg: 0.1 μM) for 0.5-4 h, and then stimulated with leptin (0.5 μg/mL) for 15 min. The phospho-JNK level was analyzed by Western blotting. **(I-L)** SH-SY5Y-Ob-Rb cells were treated with tunicamycin (Tm: 1 μg/mL) or brefeldin A (Bre: 0.03 μg/mL) for 4 h, and then stimulated with leptin (0.5 μg/mL) for 15 min. Phospho-STAT3 (Tyr705) and STAT3 levels were analyzed by Western blotting. **(M)** SH-SY5Y-Ob-Rb cells were treated with tunicamycin (Tm: 1 μg/mL, 4 h) or brefeldin A (Bre: 0.03 μg/mL, 4 h), and/or leptin for 15 min and phosphorylated tyrosine levels were analyzed by Western blotting. **(BDFHJL)** Densitometric analysis of phospho-STAT3, STAT3, phospho-ERK and phospho-JNK levels using image analyzing software. Data are expressed as the mean ± S.E. of the ratio of expression compared with DMSO or leptin alone. (*$P < 0.05$, **$P < 0.01$ statistically significant difference, n=3~4)

Fig. 3 Dose-dependence and time-course of ER stress-induced leptin resistance

(A) Dose-dependence (tunicamycin, Tm: 0.01-10 μg/mL, thapsigargin, Tg: 0.001-1 μM) and **(B)** time-course (0.5-4 h) experiments of ER stress-induced leptin resistance. HEK 293-Ob-Rb cells were pre-treated with ER stress-inducing reagents and then stimulated with leptin (0.5 μg/mL) for 15 min. Phospho-STAT3 (Tyr705) and STAT3 levels were analyzed by Western blotting. Densitometric analysis of the phospho-STAT3 (Tyr705) level was performed using image analyzing software. ER

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stress clearly inhibited leptin-induced STAT3 phosphorylation. Data are expressed as the mean ± S.E. of the ratio of expression compared with leptin alone. (*P < 0.05, **P < 0.01, ***P < 0.001 statistically significant difference, n=3)

Fig.4 4-PBA reversed ER stress-induced leptin resistance

(AB) SH-SY5Y-ObRb cells were pre-incubated with 4-PBA (10mM) for 1 h, then treated with tunicamycin (Tm: 1 μg/mL, 4 h), and then stimulated with leptin (0.5 μg/mL) for 15 min. The phosphorylation status of STAT3 was analyzed by Western blotting. 4-PBA significantly reversed ER stress-induced leptin resistance. **(B)** Densitometric analysis of the phospho-STAT3 (Tyr705) level using image analyzing software. Data are expressed as the mean ± S.E. of the ratio of expression compared with leptin alone. **P < 0.01 (significant difference, n=4~5)

Fig.5 ER stress-induced leptin resistance is mediated independently through SOCS3

(A-D) SHSY-5Y-ObRb cells were stimulated with tunicamycin (Tm: 1 μg/mL) or brefeldin A (Bre: 0.03 μg/mL) for 18 h, and then we analyzed expression levels of GRP78. We observed an increase in GRP78, indicating that ER stress was induced under the present experimental conditions. **(E-H)** SHSY-5Y-ObRb cells were stimulated with tunicamycin (Tm: 1 μg/mL), brefeldin A (Bre: 0.03 μg/mL), or leptin (0.5 μg/mL) for the indicated times and we analyzed expression levels of SOCS3. Western blotting analysis was performed using a specific antibody for SOCS3.

Although leptin increased SOCS3 levels, we did not observe SOCS3 induction in ER-stressed cells. **(I-L)** SH-SY5Y-ObRb cells were treated with tunicamycin (Tm: 1 μg/mL, 4 h) or brefeldin A (Bre: 0.03 μg/mL, 4 h) and then stimulated with leptin (0.5 μg/mL) for 15 min. Expression levels of SOCS3 or the phosphorylation status of STAT3 were analyzed by Western blotting. Although ER stress inhibited leptin-induced STAT3 phosphorylation, ER stress did not induce SOCS3. **(BDFHJL)** Densitometric analysis of GRP78 and SOCS3 levels using image analyzing software. Data are expressed as the mean ± S.E. of the ratio of expression compared with DMSO/H_2O or leptin alone. $*P < 0.05$, $**P < 0.01$ (significant difference, n=3~4)

Fig. 6 ER stress-induced leptin resistance is mediated through PTP1B

(A) SH-SY5Y-Ob-Rb cells were treated with PTP1B inhibitor for 5 h. Western blotting analysis was performed using a specific antibody for phospho-tyrosine. **(BC)** SH-SY5Y-Ob-Rb cells were pretreated with PTP1B inhibitor for 1 h and then treated with tunicamycin (Tm: 1 μg/mL, 4 h). The phosphorylation status of STAT3 was analyzed by Western blotting. PTP1B inhibitor (150 μM) significantly reversed ER stress-induced leptin resistance. **(D-G)** SH-SY5Y-ObRb cells were transfected with siRNAs (25nM). **(D)** Forty-eight h after the transfection, RT-PCR analysis was performed using specific primers for each mRNA. siRNA reduced the expression level of PTP1B. **(E)** Seventy-two h after transfection with control and PTP1B siRNA, leptin was treated for 15 min and Westren blotting analysis was performed. **(FG)** Seventy-two h after the transfection, cells were treated with tunicamycin (Tm : 1μg/mL)

for 4 h and then stimulated with leptin (0.5 μg/mL) for 15 min. Western blotting analysis was performed using specific antibodies for phospho-STAT3 (Tyr705) and STAT3. PTP1B siRNA reversed the ER stress-induced inhibition of STAT3 phosphorylation. **(CG)** Densitometric analysis of STAT3 phosphorylation using image analyzing software. Data are expressed as the mean ± S.E. of the ratio of expression compared with leptin alone. $*P < 0.05$ (significant difference, n=4)

Fig. 7 Homocysteine induced leptin resistance

(AB) HEK293-Ob-Rb cells were treated with homocysteine (Homo: 1-10 mM) for 24 h and then we analyzed the expression levels of GRP78 by Western blotting. Homocysteine increased GRP78 expression. **(CD)** HEK293-Ob-Rb cells were treated with homocysteine (1-10 mM) for 4 h and then stimulated with leptin (0.5 μg/mL) for 15 min. Phospho-STAT3 (Tyr705) and STAT3 were analyzed by Western blotting. **(EF)** HEK293-Ob-Rb cells were treated with homocysteine (10 mM) for 1-4 h and then stimulated with leptin (0.5 μg/mL) for 15 min. Phospho-STAT3 (Tyr705) and STAT3 were analyzed by Western blotting. **(BDF)** Densitometric analysis of STAT3 phosphorylation using image analyzing software. Homocysteine dose-dependently inhibited leptin-induced STAT3 phosphorylation. Data are expressed as the mean ± S.E. of the ratio of expression compared with H_2O alone. $*P < 0.05$ (significant difference, n=3)

Fig. 8 Homocysteine inhibited leptin-induced STAT3 phosphorylation *in vivo*

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(AB) Mice were pre-treated with homocysteine (Homo) for 1 h (i.p.) and then stimulated with leptin (i.v.) for 30 min. The mouse hypothalamus was isolated and the phosphorylation status of STAT3 was analyzed by Western blotting. **(B)** Densitometric analysis of STAT3 phosphorylation using image analyzing software. Homocysteine significantly inhibited leptin-induced STAT3 phosphorylation in the hypothalamus. Data are expressed as the mean ± S.E. of the ratio of expression compared with HCl alone. *$P < 0.05$ (significant difference, n=2~5)

Fig.1

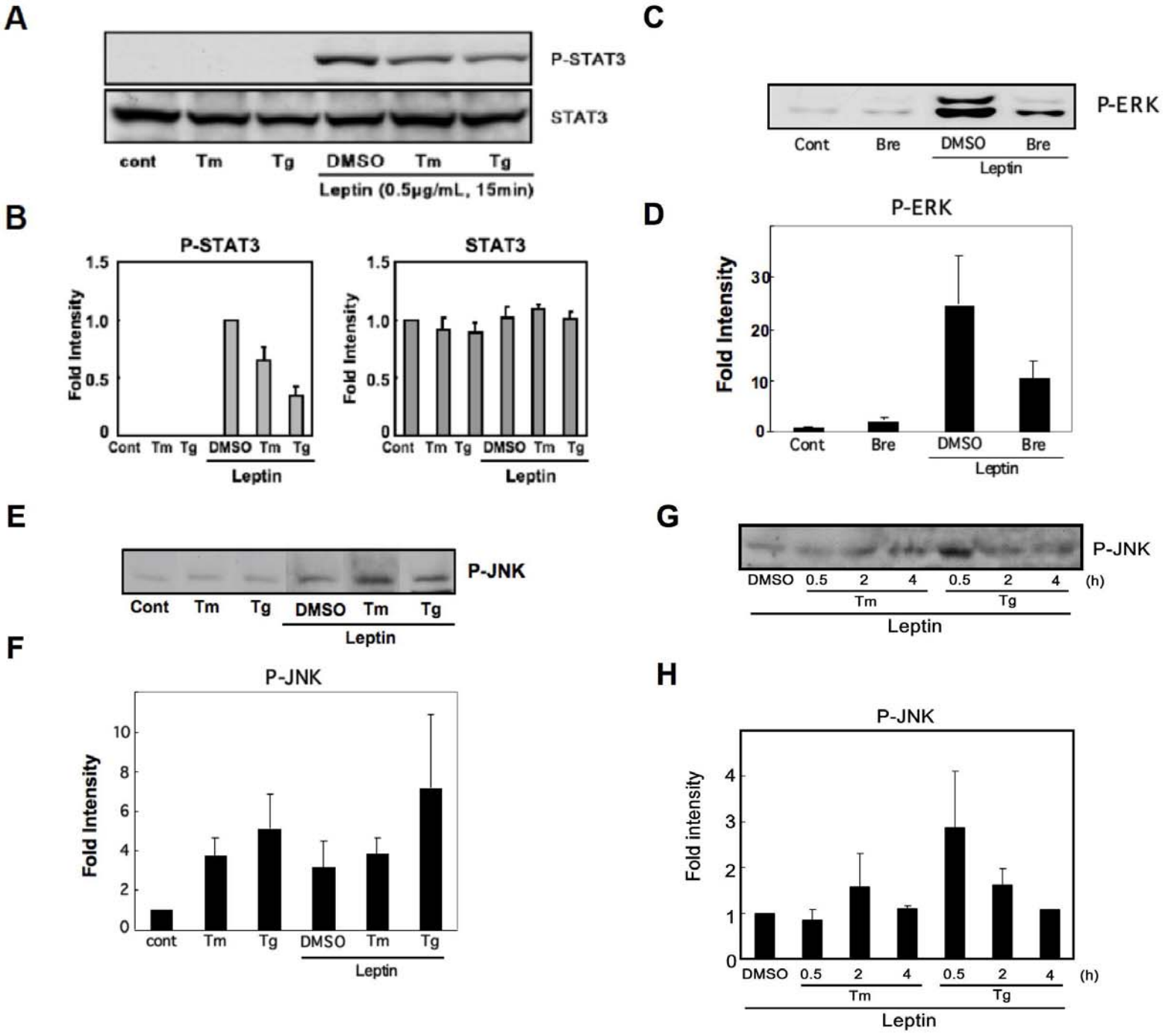

Fig.2 (A-H)

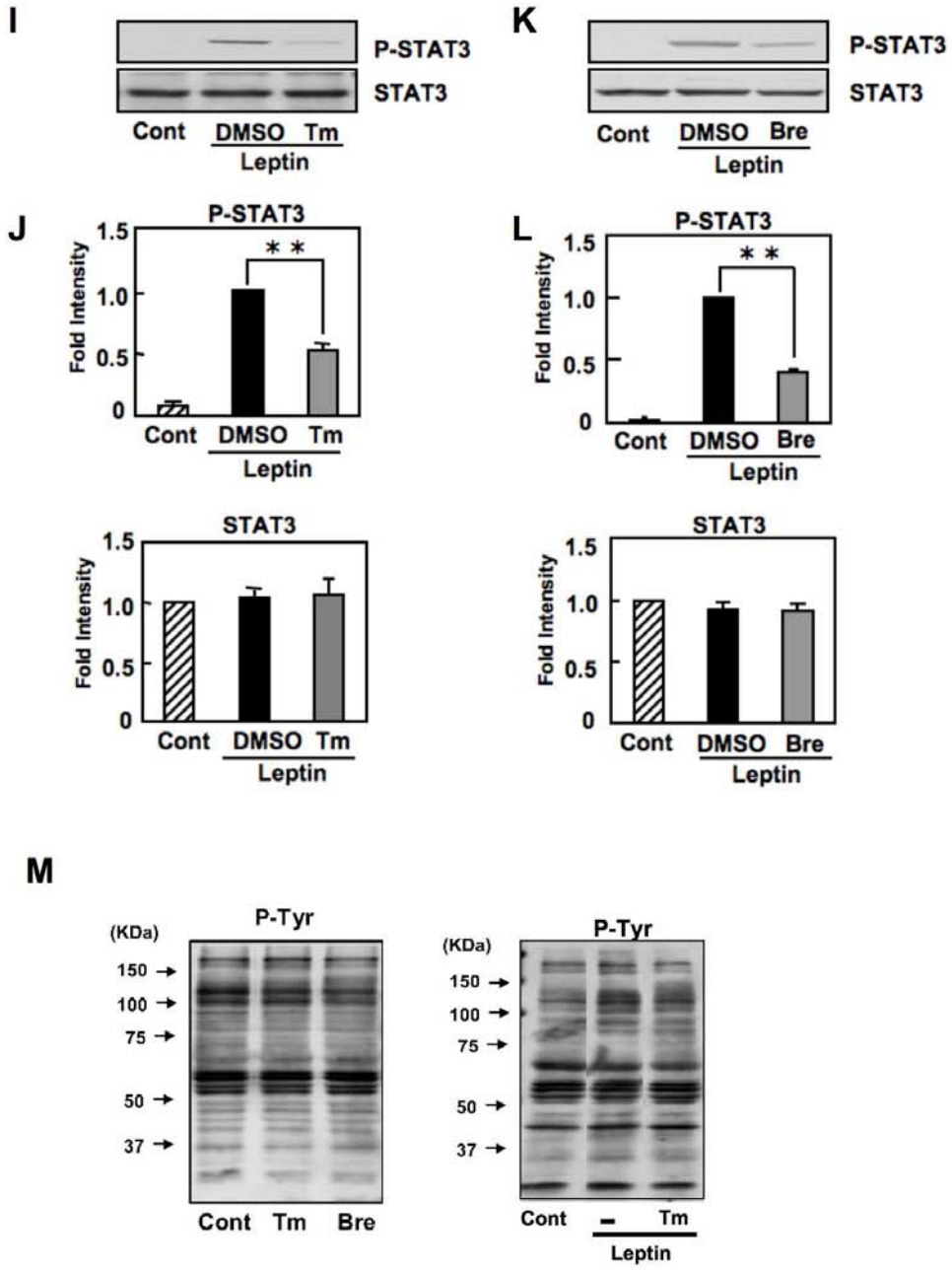

Fig.2 (I-M)

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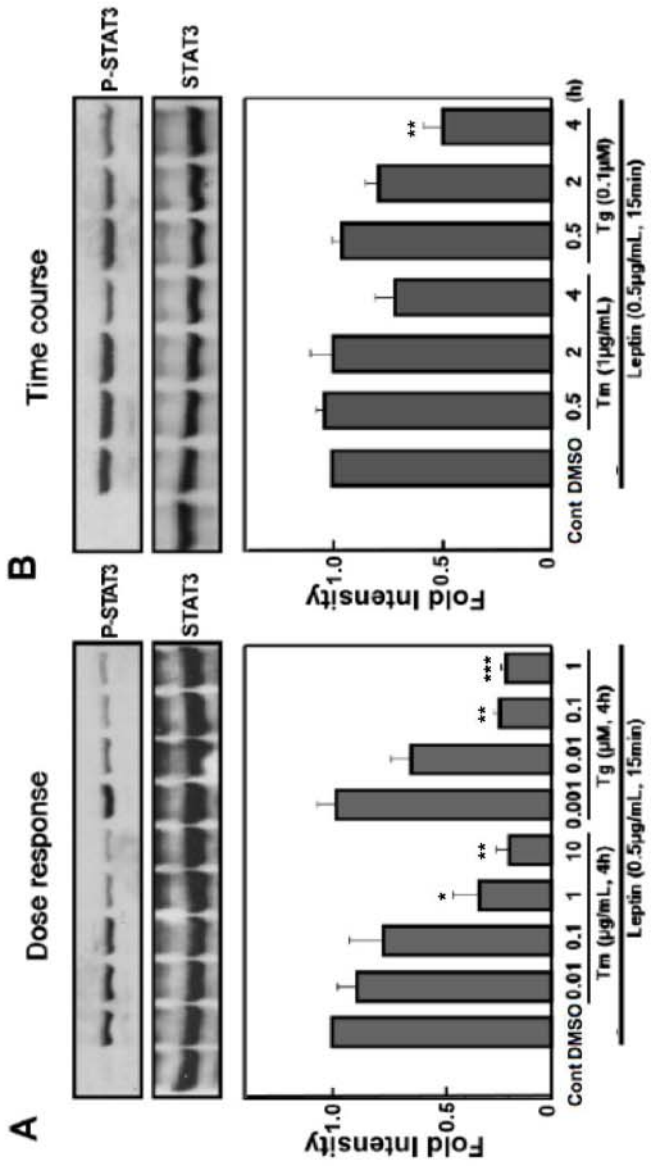

Fig.3

Fig.4

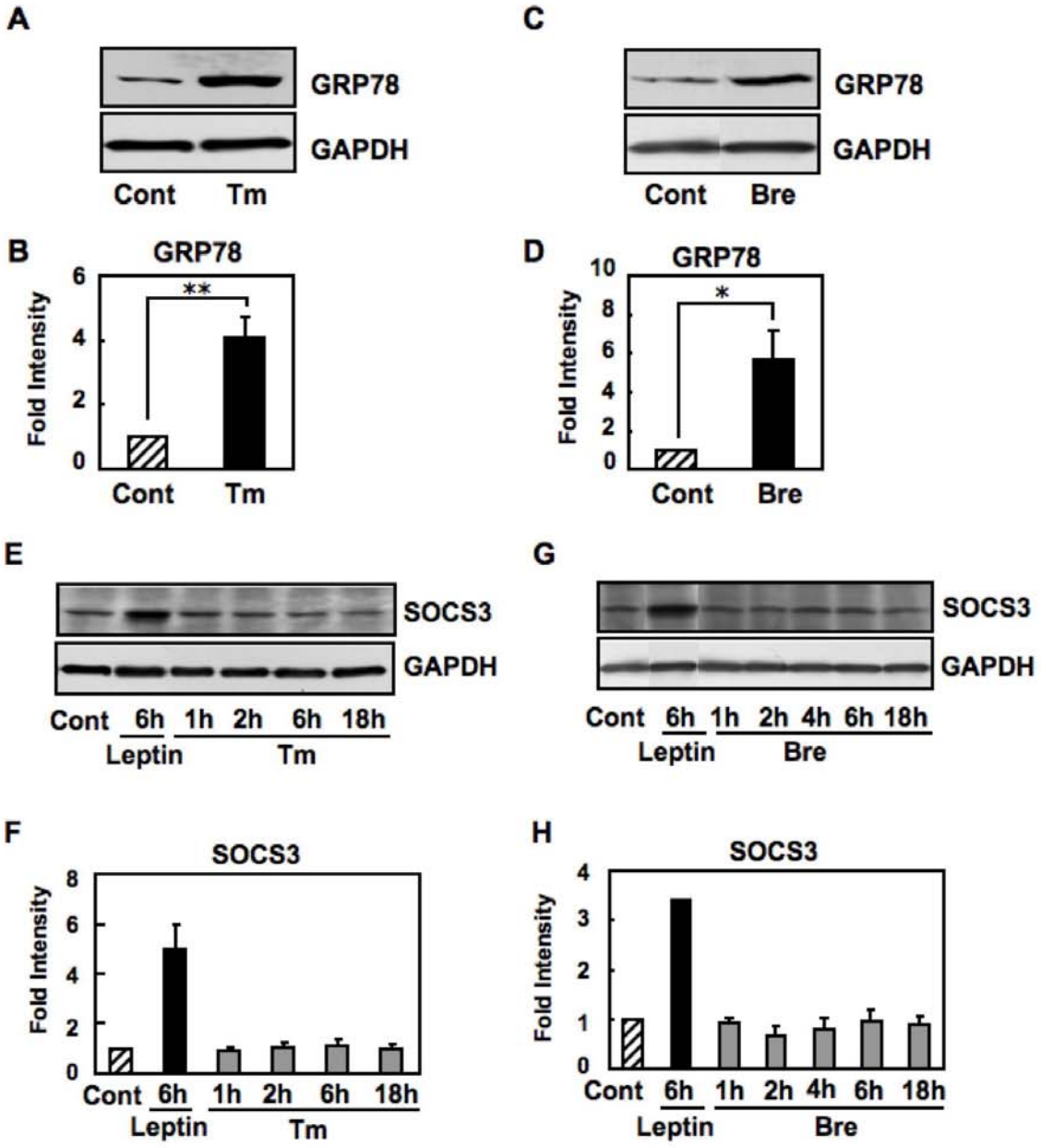

Fig.5 (A-H)

Fig.5 (I-L)

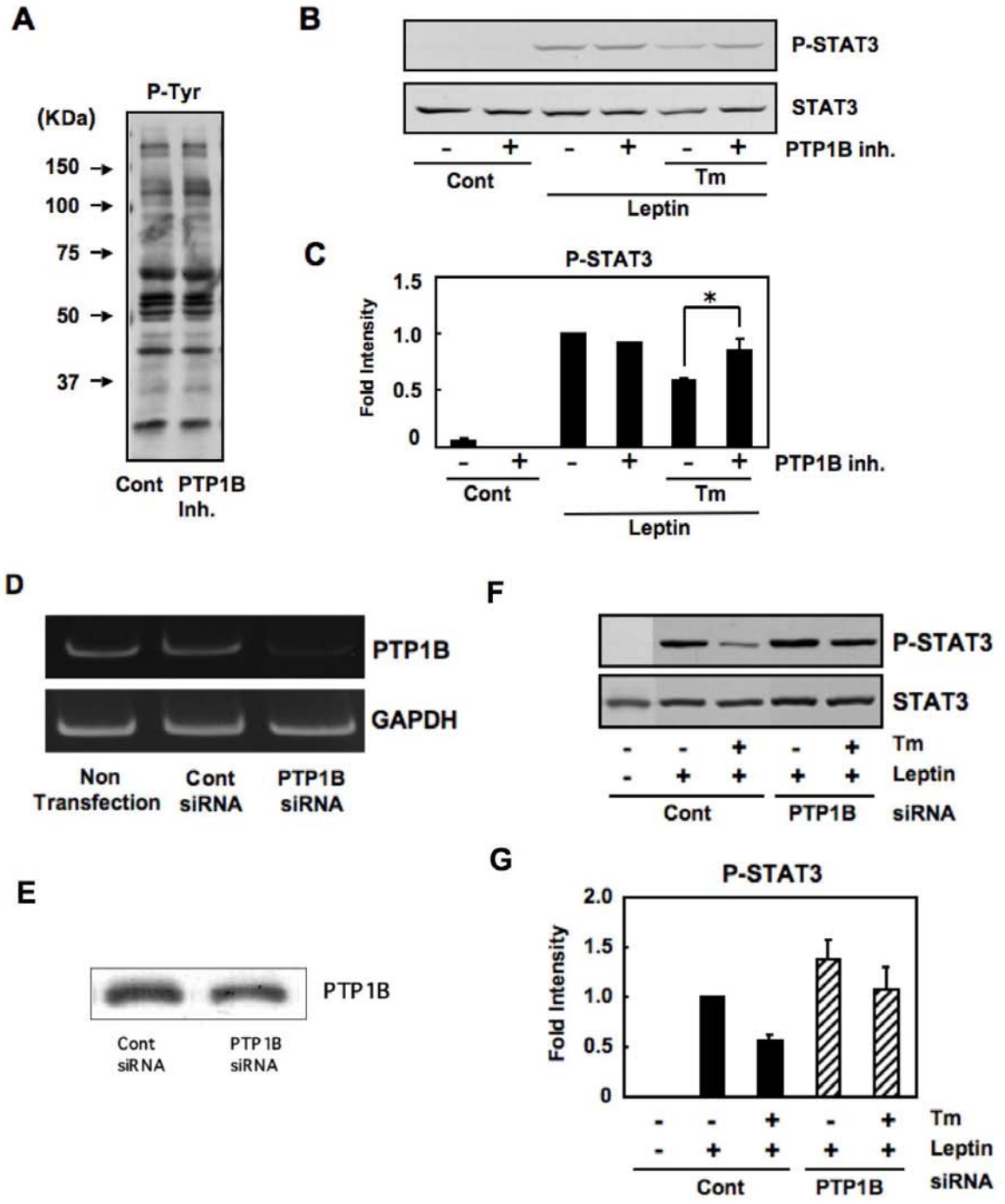

Fig.6 (A-G)

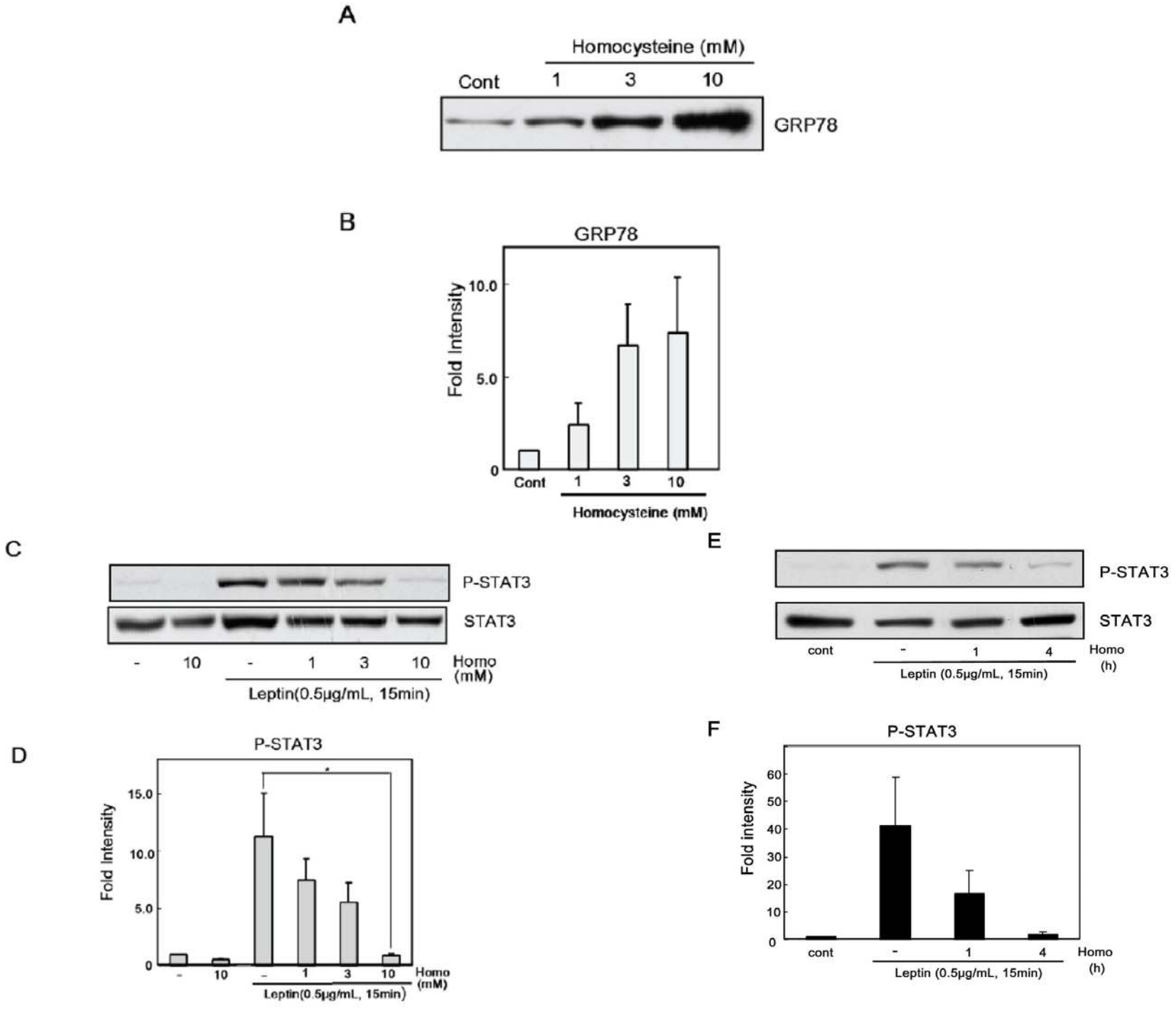

Fig.7

Fig.8