# HOMOCYSTEINE CAUSES NEURONAL LEPTIN RESISTANCE AND ENDOPLASMIC RETICULUM STRESS

**(ホモシステインは神経細胞におけるレプチン抵抗性と小胞体ストレスを誘導する)**

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## ABSTRACT

Leptin is a hormone that control energy expenditure and food intake. When leptin does not work optimally, there will be a condition called “leptin resistance”. This condition is mentioned as one of the factors causing obesity. Nevertheless, the mechanisms of leptin resistance remain unknown. In the previous study, our group reported that Endoplasmic Reticulum (ER) stress induced leptin resistance (Hosoi T., et al. 2008). ER stress occurs because of the accumulation of the unfolded proteins in the ER membrane. When this happens, the unfolded protein response (UPR) will take over in order to normalize the function of the ER. The activation of leptin signal was evaluated by Western blot analysis using a phospho (Tyr705) signal transducer and the activator of transcription factor (STAT3) antibody. ER stress blatantly inhibited leptin-induced STAT3 phosphorylation.

In the previous study, our group also reported that homocysteine might induce ER stress and caused leptin resistance. Homocysteine activates UPR in neuronal cells leading to increase the expression of GRP78/BiP as well as HERP, the ER stress response gene. We also examined whether homocysteine has a toxicity effect on the neuronal cells by CCK8 and LDH assay. We hypothesized that homocysteine caused cell death and then inhibited the JAK-STAT signaling pathway of leptin.

Together, these findings suggest that the mechanism of leptin resistance is gained from inducing ER stress.

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# CHAPTER I

# INTRODUCTION

## *A. Background*

Obesity has been a worldwide attraction since it is consorted with some metabolic disorder such as diabetes, hypertension, and dyslipidemia. Leptin is a crucial part of our body that handle food and energy consumption. Leptin is a hormone with the molecular weight 16-kDa (kilo Dalton) which playacts central mechanism in diseases especially in obesity. The action mechanism of hormone leptin is through JAK-STAT pathway. There are six leptin receptors for this mechanism action. They are LepR-a, LepR-b, LepR-c, LepR-d, LepR-e, LepR-f . These receptors are highly and mostly expressed in the hypothalamus in the brain. Receptor LepR-b is the only one which has long isoform that can be used for molecular action. Leptin binds to its receptor LepR-b, then activates JAK-STAT pathway. JAK2 will be phosphorylated and then STAT3 also will be phosphorylated. The phosphorylated STAT3 then will be translocated into the nucleus and then initiate some specific molecular mechanism. In obesity, a situation where leptin signalling pathway does not work well known as leptin resistance may befall for some reasons. Genetic problems, alteration of leptin over blood brain barrier and endoplasmic reticulum stress. We have reported that endoplasmic reticulum stress can induce leptin resistance (Hosoi et al., 2008). Thus, we will examine the mechanisms of homocysteine-induced leptin resistance through ER stress.

The endoplasmic reticulum (ER) is an organelle where protein folding and synthesis occur (Oakes SA and Papa FR., 2015). When the ER cannot handle an overwhelming amount of protein, which must be moved into the Golgi bodies for the next molecular action, homeostasis in the ER lumen will be interrupted,

and this condition is called “ER stress” (Wang M and Kaufman RJ., 2016). We and other groups have reported that ER stress may play a role in inducing leptin resistance (Hosoi et al., 2008; Ozcan L et al., 2009) and could trigger the obesity (Gruzdeva et al., 2015; Schneeberger M., 2013). Saturated fatty acid-palmitate has been suggested to advance ER stress by binding to the Toll-like receptor (TLR-4), and subsequently trigger leptin resistance resulting in the development of obesity (Oh S at al., 2019)(Milanski M et al., 2009). ER stress initiates unfolded protein response (UPR) signaling (Senft D and Ronai ZA., 2015) by promoting a molecular chaperone called glucose-regulated protein 78 (Liang W et al., 2021). Accumulation of unfolded proteins in the ER activates stress sensor proteins such as inositol-requiring kinase 1α, double-stranded RNA-activated protein kinase R-like ER kinase, and activating transcription factor 6 (ATF6). These ER stress sensor proteins activate UPR-related genes via X-box binding protein 1 or decrease the translation by activating ATF4 (Yoshida H et al., 2001). Despite the possibility that cells have the efficiency to tackle ER stress, they encounter apoptosis when their capacity is crammed by the unfolded proteins (Linton MF et al., 2016).

The homocysteine-responsive ER-resident protein (HERP), located in the ER membrane, plays an essential role in ER-associated protein degradation (ERAD) (Leitman J et al., 2014). It is also determined as a marker of ER stress-related apoptosis as it is linked to the ERAD pathway that working on ubiquitin- and proteasome-dependent degradation to get rid of the unfolded proteins (Sano R and Reed JC., 2013). HERP, which has a ubiquitin-like domain, has also been used to examine protein folding in the ER membrane since Herp has been suggested to ameliorate the folding of ER proteins and to dampen ER protein load (Slodzinski H et al., 2009). When ER stress occurs, HERP is highly induced (Ma Y and Hendershot LM., 2004).

Homocysteine (Hcy) is formed by methionine metabolism (Finkelstein JD and Martin JJ., 2000) through the chronological synthesis of S-

adenosylmethionine and S-adenosylhomocysteine. Several reports have suggested the involvement of homocysteine and its metabolites in obesity. Changes in plasma betaine, choline, and glycine in the development of obesity may result in changes in the homocysteine/methionine metabolism (Sivanesan S et al., 2018)(Chang TY et al., 2022; Nathanielsz PW et al., 2015; Alves A et al., 2019). Plasma homocysteine showed significant accumulation in obesity (Lima A et al., 2019; Narin F et al., 2005). Homocysteine has been reported to participate in DNA methylation in the methionine cycle, which is involved in epigenetic modification of the genome (Wang WM and Jin HZ., 2017; Jin Y., 2011).

Our previous findings and those of others have found ER stress to be one of the factors inducing leptin resistance. In addition, we previously reported that homocysteine may induce leptin resistance using HEK293 cells expressed leptin receptors (Hosoi T et al., 2008). However, the mechanism of homocysteine-induced leptin resistance remains unclear. Furthermore, it is unknown whether homocysteine can cause leptin resistance in neuronal cells.

## *B. Statement of Problem*

Obesity appears to be an urgent problem. According to WHO, the number of people with obesity has advanced from 4% to 18% from 1975 to 2016 and about 600 million of people are obese in the world. Leptin is a hormone that be in charge of food and energy control. The latest research found that leptin resistance becomes one of the causes of obesity. Our laboratory also has found that leptin resistance could happen because of endoplasmic reticulum (ER) stress. Homocysteine as an inducer of ER stress become our focus in this research. Because homocysteine could inhibit methylation reaction, then we assume that leptin resistance also happens not just because of ER stress but also inhibition of methylation reaction.

## *C. Purpose of Study*

Homocysteine was reported that it could cause leptin resistance. Thus, our purpose for this research is to clarify the detailed the mechanism of homocysteine-induced leptin resistance.

## *D. Hypothesis*

If we treat the neuronal cells with homocysteine (various concentrations) and stimulate them using leptin, then cells with a high concentration of homocysteine will potently inhibit leptin signal compared to low concentration of homocysteine. If homocysteine inhibits leptin signal, it may be because of endoplasmic reticulum (ER) stress and methylation. Therefore, if we detect the expression of ER stress response gene-HERP, by western blot analysis, we can understand whether homocysteine will increase ER stress in our neuronal cellular model.

# CHAPTER II

# LITERATURE REVIEW

## *A. Leptin*

### 1. Leptin signaling

Leptin is a hormone excreted from the adipose tissue (Zhao S et al., 2020) with its crucial tasks especially in food repression, energy regulator, and the other molecular mechanisms (Katsiki et al., 2018). Leptin acts by binding to its receptor (LepRs), these receptors are predominantly abundant in the brain especially in the hypothalamus region. There are six isoforms of leptin receptors. They are ObRa, ObRb, ObRc, ObRd, ObRe and ObRf. Among the receptors, LebR-b is the only one that has a long isoform and is widely and mostly expressed in the hypothalamus (Perez A et al., 2018). It drives the activation of Janus kinase and signal transduction, and activators of transcription known as the JAK-STAT signaling pathway. JAK-STAT is being the main pathway in leptin signaling (Hosoi et al., 2016). When leptin binds to its receptor, JAK kinases become activated and phosphorylated. After the phosphorylation of JAK, then STAT is also activated and phosphorylated then becomes dimerized. The dimerized STAT is then translocated into the nucleus and triggers molecular actions (Xin P et al., 2020).

There are two proteins that regulate leptin signaling, they are suppressors of cytokine signaling 3 (SOCS3) and protein tyrosine phosphatase 1B (PTP1B). SOCS3 has been shown to reduce appetite and PTP1B to reduce body weight. The alterations of SOCS and PTP1B are shown to be involved in leptin resistance (Izquierdo AG et al., 2019) which indicates a negative feedback action on the leptin receptor (Daniel V et al., 2006; Vaisse C et al., 1996).

### 2. Leptin resistance

A state of leptin resistance is known where leptin is ineffectively working and this state could be caused by some reasons such as molecular alterations and gene mutation (Gruzdeva et al., 2019), also endoplasmic reticulum stress (Hosoi T et al.,2008; Ozcan et al., 2009; Perez et al., 2020; Genchi VA et al., 2021; Cui H et al., 2022).

Leptin resistance is strongly suggested as the condition of the impairment of leptin's signaling (Obradovic M et al., 2021). The impairment of leptin's signaling may be caused by the overexpression of negative regulators of leptin such as SOCS3 and PTP1B. The overexpression of these negative regulators triggers to inhibit JAK-STAT3 signaling.

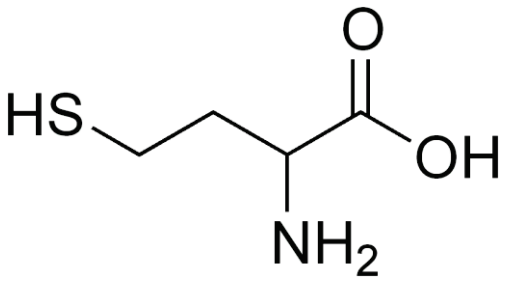
## *B. Endoplasmic Reticulum Stress*

The Endoplasmic reticulum (ER) is known as an organelle for protein folding (Chen X et al., 2020). Endoplasmic reticulum stress (ER stress) is commonly caused by the limited folding ability of the ER against the accumulation of the unfolded proteins (Cakir I et al., 2019). ER plays role in activating the unfolded protein response (UPR) signaling. UPR decreases the number of unfolded proteins and drives autophagy and ER-associated degradation (ERAD). There are three branches of UPR, they are inositol requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6). Under normal conditions, these branches are inactive upon binding to the ER protein chaperone, GRP78. While in the ER stress condition, GRP78 activates these branches (Lebeaupin et al., 2018). IRE1 triggers the activation of X-box binding protein 1 (XBP1) and produces XBP1 spliced (Guzel et al., 2017). PERK acts by phosphorylating eukaryotic translation initiation factor 2 subunit alpha (eIF2α). After phosphorylating eIF2α, there are two kinds of mechanisms: attenuation of translation and selective translation. When the translation is attenuated, the protein accumulation is decreased. When a selective translation is initiated, it encodes activating

transcription factor 4 (ATF4). Next, ATF4 will be translocated into the nucleus and triggers some molecular events (Hetz C et al., 2020). The last branch of UPR is ATF6. During ER stress, ATF6 is translocated into the Golgi and cleaved by site-1 and site-2 proteases (S1P and S2P) then strengthens the endoplasmic reticulum-associated protein degradation (ERA) pathway (Hetz C et al., 2017).

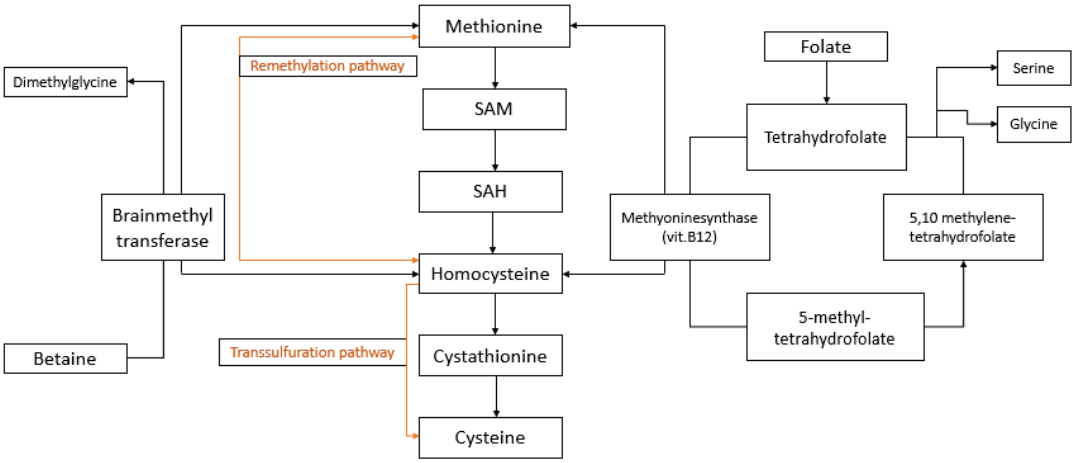
### *C. Homocysteine*

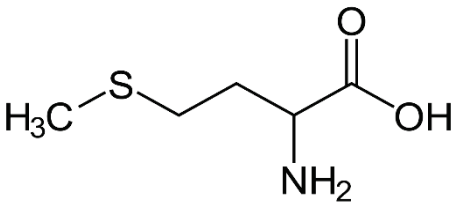
Homocysteine (Hcy) is an amino acid protein that comes from two pathways: remethylation and transsulfuration (Jakubowski., 2019). Homocysteine is remethylated to form methionine by two mechanisms. The first is by using vitamin B and folate. This folate donor a methyl group to Hcy which is catalyzed by the vitamin B12-dependent enzyme methionine synthase. The second is using betaine as a methyl group donor (Škovierová H et al., 2016). While in the transsulfuration pathway, cysteine is synthesized (Portillo et al., 2020).



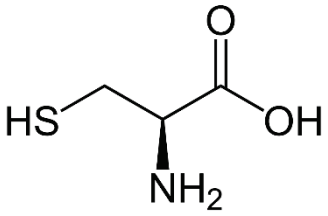
***Homocysteine structure***

Homocysteine is converted to methionine by the demethylation pathway. The other pathway is the transsulfuration pathway (Van Guldener C et al., 2006) which is mainly found in the liver, kidney, small intestines and pancreas (Madalena B et al., 2017).




***Methionine structure***




***Cysteine structure***

***Homocysteine metabolism***. *Methionine is converted to homocysteine as a result of methylation reactions, which is activated by ATP to form S-adenosyl-methionine (SAM). Then SAM is converted to S-adenosyl-homocysteine (SAH) as a result of methyl group transfer. The concentration of homocysteine is maintained by two pathways; the remethylation where homocysteine is converted back to methionine and transsulfuration where homocysteine is converted to cystathionine in order to form cysteine.*

There are two metabolites of homocysteine. They are S-adenosyl-homocysteine (SAH) and S-adenosyl-methionine (SAM). SAM is a fundamental co-substrate that is used by DNA methyltransferase enzymes in bringing the group of methyl to DNA and the product from this reaction is known as S-adenosyl-homocysteine (SAH) (Mandaviya PR et al., 2014). SAM and SAH concentrations are important for cellular methylation status (Chen NC et al., 2010). A decrease in SAM: SAH ratio is in the presence of an

increase or no change in SAM, but a significant increase in SAH is associated with hypomethylation and a decrease the methyltransferases activity (Yi P et al., 2000). Several studies found that there is an association of homocysteine and obesity (Vural Y et al., 2014).

Homocysteine is also known as ER stressor proved by increased expression of ER major chaperone, GRP78/BiP, and enhanced phosphorylation of PERK, one of ER stress markers (Wang XC et al., 2015).

### *D. EZH2 inhibitor*

Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that widely targeted in cancer therapy (Hanaki S and Shimada M., 2021). EZH2 that mostly expressed in the proliferating cells, plays a crucial role in cell cycle (Nutt SL et al., 2020). The change of EZH2 function from histone methyltransferase to the non-histone methyltransferase inevitably methylates STAT3 and induce neuroendocrine differentiation (Luo j et al., 2019). Wang J et al also have reported that the inhibition of EZH2 can lead to the decrease of atherosclerosis through the inhibition of STAT3.

Anomalous of IL-6 or STAT3 signalling in the cancer cells have exposed as the beginning of the development of cancer (Pan YM et al., 2016). The decrease of STAT3 triggers apoptosis by inhibiting EZH2 and the relation between STAT3 and EZH2 may be underlying the mechanism by which STAT3 controls the proliferation of cells. In line with the previous sentences, Zhao Y et al., 2016 has also reported that the overexpression of STAT3 may retrieve the inhibition of EZH2-restrained the proliferation of cancer cells.

Since homocysteine is reported to inhibit methylation (Jamaluddin MD et al., 2007) and the EZH2 can methylate STAT3 through the suppression of STAT3 (Kim E et al., 2013), we also would like to examine whether protein methylation has possible involvement in the homocysteine-induced leptin resistance.

# CHAPTER III

# RESEARCH METHODS

## *A. Materials and reagents*

Homocysteine (Hcy) and methionine was obtained from Sigma-Aldrich Cheme (St. Louis, US), cysteine was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan), anti-phospho STAT3 was obtained from Cell Signaling Technology (Danvers, US), anti-STAT3 was obtained from Santa Cruz Biotechnology (Dallas, US), and anti-HERP was obtained from Proteintech (Sank Leon-Rot, Germany).

## *B. Cell Culture and Stimulation*

### 1. SHSY5Y-ObRb

Human neuroblastoma cell lines: SH-SY5Y neuroblastoma cells, which stably express Ob-Rb leptin receptor (SH-SY5Y Ob-Rb cells), was established previously (Hosoi et al., 2008). SH-SY5Y Ob-Rb cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37℃ in humidified 5% $CO_2$ and 95% air.

### 2. HEK293T cell line

Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37℃ in humidified 5% $CO_2$ and 95% air.

3. **Cell Stimulation**

Homocysteine was dissolved directly into the culture medium. The cells were stimulated with homocysteine for 4 hours by replacing the medium containing homocysteine with or without stimulator.

## *C. Cell Harvest*

Cells were washed with ice-cold Phosphate-Buffer-Saline and lysed by lysis buffer containing 1M HEPES-NaOH (pH 7.5), 1.54M NaCl, 0.5M EDTA, 0.1M $Na_3VO_4$, 0.5M NaF, 20% NP-40, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 0.1 Phenylmethylsulphonyl Fluoride. The lysates were centrifuged at 15000 rpm, 4$^oC$ for 20 minutes. After centrifuging, the supernatants were collected. After that, the samples were boiled with Laemmli buffer for 3 minutes.

## *D. Bradford Assay*

The concentration of collected proteins from the previous centrifugation was measured using Bradford Assay.

## *E. Western Blot*

Samples were fractioned by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-Page) and transferred at 4$^oC$ to nitrocellulose membranes. Then, the membranes were incubated with anti-phospho STAT3 (Tyr705; dilutions 1:2000; Cell Signalling Technology), anti-STAT3 (dilution 1:1000; Santa Cruz Biotechnology), anti-phospho JAK2 (Tyr1007/1008; dilution 1:1000; Cell Signalling Technology), anti-JAK2 (dilutions 1:1000; Cell Signalling Technology), anti-KDEL (dilutions 1:1000; Enzo Life Sciences), anti-GAPDH (dilutions 1:1000; Proteintech), and anti-HERP (dilutions 1:1000; Proteintech) antibodies, followed by anti-horseradish peroxidase-linked

antibody. Then, peroxidase binding was detected by using chemiluminescence reagents.

### *F. CCK-8 Assay*

SHSY5Y-ObRb were cultured in 96-well plate and treated with homocysteine (Hcy) with concentrations at 1 mM, 3 mM and 10 mM then incubated for 4 hours. Then, cells were added with CCK8-solution, incubated at 37ºC, 5% $CO_2$ for 1 hour and then measured at 450 nm.

### *G. Cytotoxicity LDH Assay*

SHSY5Y-ObRb cells were cultured in the 96-well plate, with 10000 cells/well. Then, cells were treated with homocysteine 1 mM, 3 mM, and 10 mM for 4 hours. Then, cells were incubated for 24 hours and added lysis buffer, then incubated again for 30 minutes. After that, each well was added with 100 $\mu$l working solution and incubated at 37ºC, 5% $CO_2$ for 30 minutes. Then, well were added with 50 $\mu$l stop solution and measured at 490 nm.

### *H. Statistics*

Results are described as the mean $\pm$ S.E of the value. Statistical analyses were performed using the Dunnett test or Turkey test.

# CHAPTER IV

# RESULTS

## *A. Homocysteine suppresses of leptin receptor induced signalling pathway*

To assess the effect of homocysteine on leptin signaling, we pre-treated the SH-SY5Y-Ob-Rb, human neuroblastoma cell line, with homocysteine at 3 and 10 mM and analyzed the phosphorylation of STAT3 induced by leptin (0.01 µg/mL, equal to 0.6 nM). As expected, leptin treatment caused an increase of STAT3 phosphorylation in these cells. Pretreatment with 3 mM homocysteine slightly, but not significantly, inhibited leptin-induced STAT3 phosphorylation (p-value = 0.44), while 10 mM homocysteine significantly inhibited leptin-induced STAT3 phosphorylation (*p< 0.05) (Figs 1A and 1B).

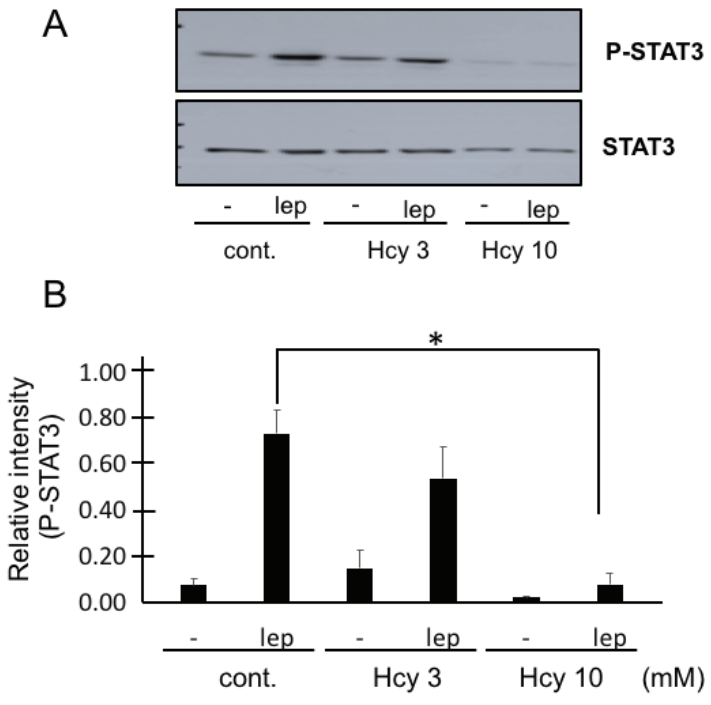



***Fig 1A-B. Homocysteine inhibited leptin-induced STAT3 phosphorylation.*** SHSY5Y Ob-Rb cells were pretreated with homocysteine (Hcy; 3 and 10 mM) for 4 h and then stimulated with leptin (Lep; 0.01 µg/mL) for 15 min. Leptin-induced STAT3

phosphorylation was analysed by immunoblot. Statistical analysis used Dunnett's post-hoc test following one-way ANOVA; *p< 0.05, n = 3-5.

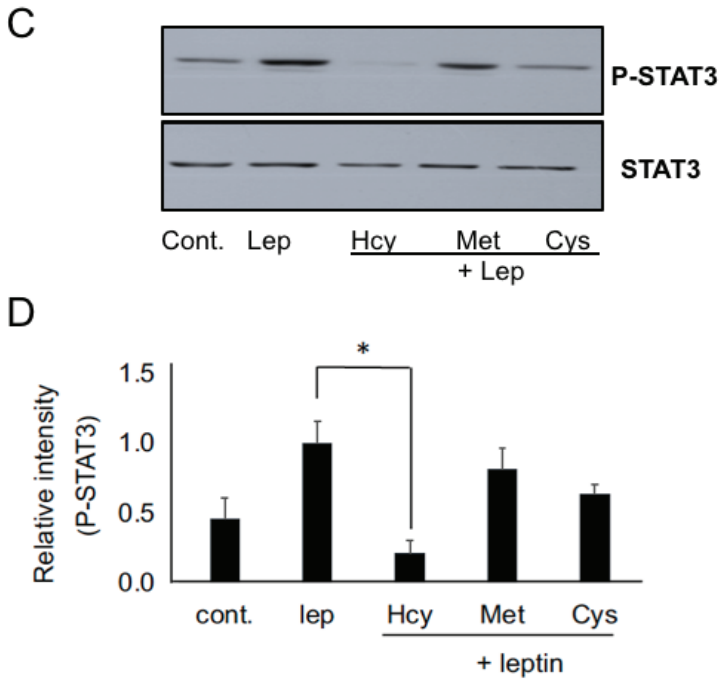



***Fig 1C-D. Methionine and cysteine were slightly inhibited leptin-induced STAT3 phosphorylation.*** SHSY5Y Ob-Rb cells were treated with homocysteine (Hcy, 10 mM), methionine (Met, 10 mM) and cysteine (Cys, 10 mM) for 4 h and then stimulated with leptin (Lep; 0.01 µg/mL, 15 min). Leptin-induced STAT3 phosphorylation was analysed by western blot analysis. Statistical analysis used Turkey's post-hoc test following one-way ANOVA; *p < 0.05, n = 3- 5.

To account for the chemical specificity of these effects, we next evaluated the impact of other compounds related to homocysteine, i.e., methionine (10 mM) and cysteine (10 mM), which have a structural formula similar to homocysteine. Whereas methionine and cysteine did not inhibit leptin-induced phosphorylation of STAT3 significantly, homocysteine significantly inhibit leptin-induced phosphorylation of STAT3 (*p<0.05) (Figs 1C-D). These results therefore, suggest that homocysteine specifically inhibits the leptin-induced STAT3 signaling compared to methionine and cysteine in a neuronal context.To know the effect of homocysteine on leptin signaling, we pre-treated the

SHSY5Y-ObRb cells with homocysteine at the concentration of 3 mM and 10 mM for 4 hr. Then, we examine the phosphorylation levels of STAT3. As we expected, homocysteine induced leptin resistance. Especially, homocysteine 10 mM as the representative of the higher concentration used in the cell line, significantly decreased leptin-induced phosphorylation of STAT3. While homocysteine 3 slightly decreased leptin-induced phosphorylation of STAT3.

### *B. Homocysteine induces endoplasmic reticulum stress*

Next, we evaluated the mechanism of homocysteine-induced leptin resistance. One possible explanation is that homocysteine induces leptin resistance through the activation of ER stress. We here found that homocysteine increased the expression level of the ER stress response gene, HERP at 4 and 8 hours, by 4-fold in SHY5Y cells. We analysed whether homocysteine-induced HERP, known to be involved in ER stress.

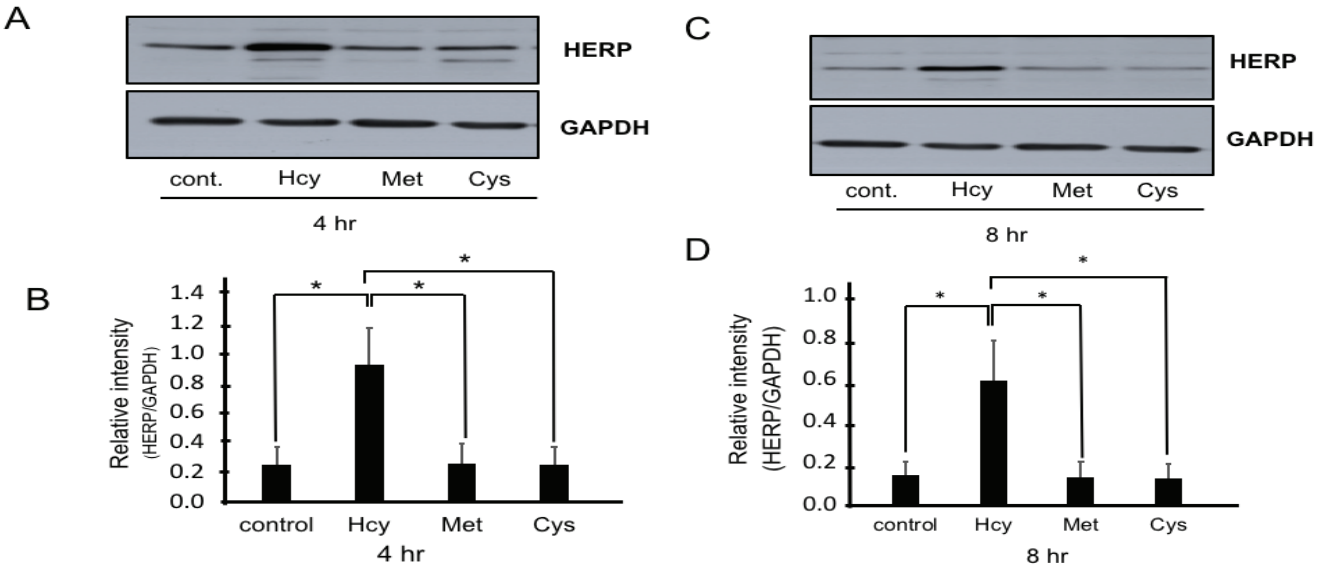



***Fig 2A-D. Homocysteine induced endoplasmic reticulum stress.*** (A-D) SHY5Y-ObRb cells were treated with homocysteine (Hcy), methionine (Met), and cysteine (Cys) for 4 and 8 hours at 10 mM, and the expression levels of ER stress response gene, HERP, was analyzed by Dunnett test. *$p < 0.05$, $n = 5$.

In contrast, we did not detect an increase in HERP expression after treatment with cysteine and methionine (*$p < 0.05$, $n = 5$) (Figs 2A - 2D). Thus, we suggest that homocysteine may participate in leptin resistance by inducing ER stress.

## *C. Homocysteine has no effect on the IL-6 signalling pathway*

To assess whether homocysteine has a specific impact on leptin signaling, we examined the effect of homocysteine on interleukin-6 (IL-6)-induced STAT3 pathway. We treated cells with homocysteine (10 mM, 4 h) and analysed IL-6 (100 ng/mL)-induced STAT3 phosphorylation in HEK293T cell line. We found that homocysteine did not affect IL-6-induced STAT3 signaling (Figs 3A and 3B).

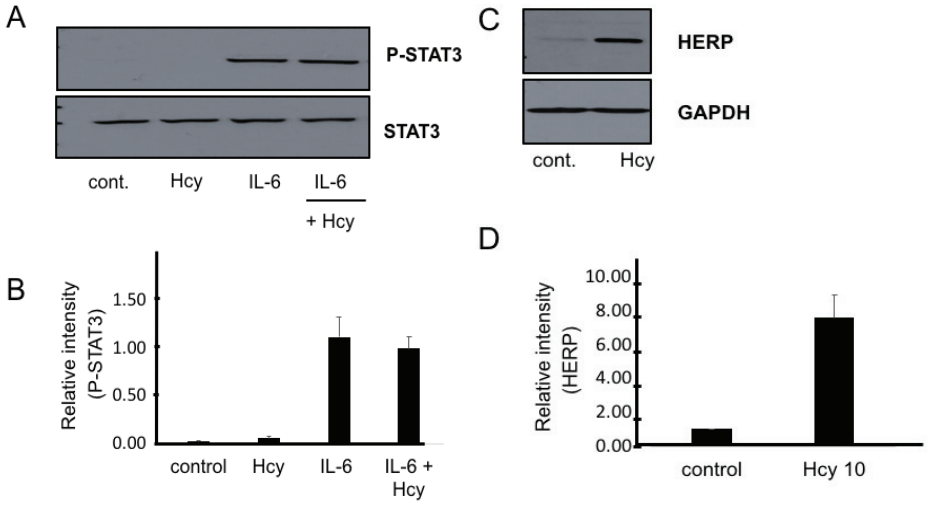



***Fig 3. Homocysteine has no impact on IL-6 signaling pathway****. (A-B) HEK293T cells were treated with homocysteine (Hcy) for 4 hours at 10 mM, then stimulated with IL-6 (100 ng/mL) for 15 minutes. The levels of phospho-STAT3 were analysed. n=5. (C-D) HEK293T cells were treated with homocysteine (Hcy) for 4 hours at 10 mM, then stimulated with IL-6 (100 ng/mL) for 15 minutes. The levels of HERP were analysed. n=5.*

On the other hand, homocysteine can inhibit leptin-induced STAT3 phosphorylation in HEK293 cells stably transfected with Ob-Rb. Interestingly, the expression level of HERP as an ER stress response gene at 4 hours was significantly increased by homocysteine in HEK293T cells (Figs 3C and 3D), suggesting that homocysteine also likely contributes to ER stress in the HEK293T cell line, similarly to the neuronal cells. Therefore, the impact of homocysteine on leptin-induced STAT3 phosphorylation appears to be specific to leptin receptor signaling since homocysteine, by inducing HERP expression, did not affect STAT3 phosphorylation mediated by IL6.

## *D. Effect of homocysteine on the number and viability of cells*

Because the previous results showed that homocysteine caused leptin resistance induced by endoplasmic reticulum stress, we investigated whether homocysteine at such a high concentration could affect the number and viability of cells. Thus, we performed a CCK8 assay to confirm the effect of homocysteine on the number and viability of cells.

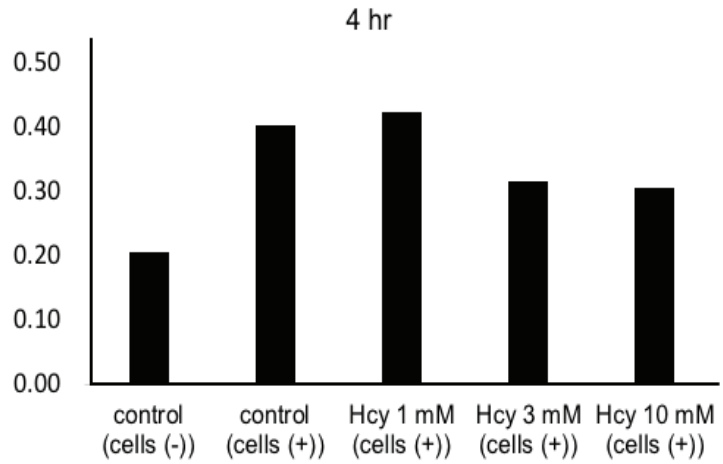



***Fig 4. Homocysteine causes cell death***. *SHSY5Y-ObRb were cultured in 96-well plate and treated with homocysteine (Hcy) with concentrations at 1 mM, 3 mM and 10 mM*

*then incubated for 4 hours. Then, cells were added with CCK8-solution, incubated for 1 hour and then measured the absorbance at 450 nm. n=3.*

## *E. Toxicity effect of homocysteine on cells*

As our previous results showed that homocysteine caused cell death, then we tried to know how much damage or toxicity that homocysteine may cause to cells. Thus, we performed a cell toxicity experiment; LDH assay to confirm this.

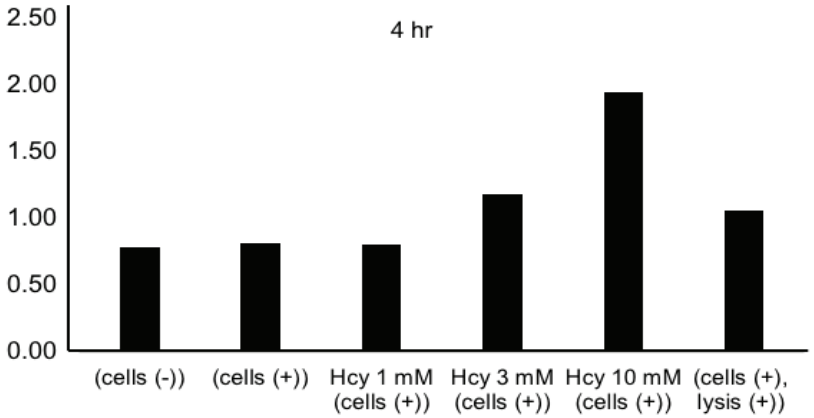



***Fig 5. Homocysteine seemed to cause damage to cells**. SHSY5Y-ObRb were cultured in 96-well plate and treated with homocysteine (Hcy) at 1 mM, 3 mM and 10 mM and absorbance were measured at 490 nm. n=3.*

## *F. No impact of EZH2 inhibitors on leptin resistance*

To examine whether the activity of EZH2 in obesity is decreased or not, we pre-treated the SHSY5Y-expressing ObRb cell lines with EZH2 inhibitors (GSK126 (2 and 10 μM) and EPZ005687 2 and 10 μM)) at 8 hr and 24 hr. Then, we examined the phosphorylation levels of STAT3.

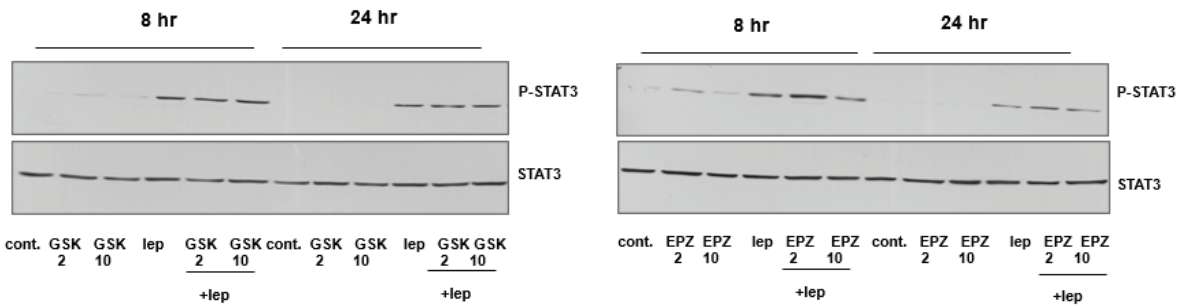



***Fig 6. Epigenetic events may has no involvement in leptin resistance.*** *SHSY5Y-ObRb were cultured in 10-cm dish and treated with GSK126 and EPZ005687 with concentrations at 2 mM, and 10 mM, n=3.*

As the result, the leptin signal was not inhibited by the treatment of EZH2 inhibitors. The expression of P-STAT3 was increased after leptin stimulation. We suggested that EZH2 inhibitors have no involvement in leptin resistance-induced obesity. Then, we examined the expression level of the ER stress response gene, HERP to know whether this protein methylation has an involvement in ER stress.

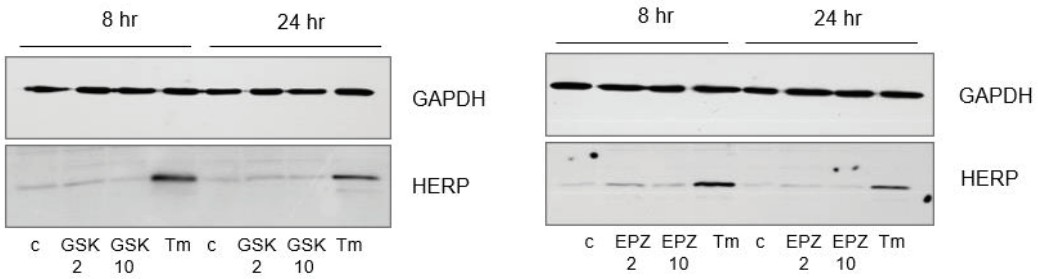



***Fig 7. Epigenetic events may have no involvement in ER stress****. SHSY5Y-ObRb were cultured in 10-cm dish and treated with GSK126 and EPZ005687 with concentrations at 2 mM, and 10 mM, n=3.*

As the result, both of EZH2 inhibitors showed no involvement in ER stress, While, only tunicamycin (ER stress inducer) increased the expression levels of

HERP. In conclusion, this epigenetic event may have no direct involvement in homocysteine-induced leptin resistance.

## *G. Discussion*

Leptin is a hormone that plays roles in food intake and energy expenditure. Leptin binds to its receptor Ob-Rb and then activates the JAK-STAT signaling pathway. In terms of leptin resistance, JAK-STAT signaling pathway does not work properly, resulting in obesity. Some causes reported to induce leptin resistance such as impairment of leptin transport in the blood-brain barrier (Balland E et al., 2014; Duquenne M et al., 2021; Banks WA et al., 2003; Liu H et al., 2021), a decrease of the leptin receptor-ObRb in the cell surface (Roujeau C et al., 2019; Vauthier V et al., 2014; Guo DF et al., 2016, Wijesuriya TM et al., 2021; Mazor R et al., 2018, Münzberg H et al., 2004; Gao Q et al., 2004; Myers MG et al., 2010), and the induction of endoplasmic reticulum stress (ER stress) (Hosoi et al.,2008; Ozcan L et al., 2009).

Homocysteine is a sulfur-containing, non-protein amino acid that naturally exists in the bloodstream. Its concentration is controlled by the re-methylation and transsulfuration pathways. The re-methylation pathway involves conversion of homocysteine to methionine, whereas the transsulfuration pathway involves conversion of homocysteine to cystathionine to form cysteine.

In this study, we discovered a functional link between homocysteine and leptin resistance in neuronal cells that was previously unknown. Homocysteine inhibits leptin signaling (inhibition of leptin-induced STAT3 phosphorylation and subsequent STAT3 transcriptional activity), suggesting that homocysteine may be involved in the leptin resistance phenomenon (Fig. 1). The mechanism by which homocysteine inhibits the leptin-induced STAT3 signalling pathway may involve the ER stress response gene, HERP, whose expression is increased

upon homocysteine treatment. Thus, homocysteine may induce ER stress leading to leptin resistance in neuronal cells (Fig. 2).

HERP (homocysteine-induced endoplasmic reticulum protein) is a 54 kD membrane protein, encoded by homocysteine-inducible and ER stress-inducible ubiquitin like domain member 1 (Herpud-1), localized in the endoplasmic reticulum (Nogalska A et al., 2006), interacts with the ubiquilin family for degradation through ERAD (ER-associated protein degradation) pathway (Kim TY et al., 2008). Homocysteine induces ER stress is followed by the production of homocysteine-inducible ER protein or HERP (Oka Y et al., 2007)(McLaughlin M et al., 2010). Then, this HERP protein is specific to homocysteine.

Next, according to our result of IL-6-induced phosphorylation of STAT3 in HEK cells, leptin signalling pathway is the specific pathway for homocysteine-induced leptin resistance (Fig.3).

CCK8 assay and LDH assay are two ways to examine whether a compound has a toxicity and effect on the viability of cells. In our result, homocysteine 10 mM (that may be high to be used in the experiment), showed to decrease the phosphorylation of STAT3 stimulated by leptin. The decrease of P-STAT3 seemed to caused by the cell death based on our CCK8 assay and LDH assay results (Fig.4 and 5).

Enhancer of zeste homolog 2 (EZH2) potential molecular biomarkers for tumor progression that exerts oncogenic effect via methyltransferase activity (Zhang J et al., 2021) in the regulation of gene expression. EZH2 regulates the expression of its target genes by the trimethylation of Lys-27 in histone 3 or methylates non-histone proteins such as STAT3 in the STAT3 signalling pathway (Wang H., 2022). As for our results, the EZH2 inhibitors did not show the decrease of the leptin-induced phosphorylation of STAT3 (Fig. 6).

Another compound that commonly used as ER stress inducer is tunicamycin (Zhang X et al., 2014) (Wu J et al., 2018)(Yemenici M et al., 2022). Thus, to know whether HERP protein is regulated by ER stress, then stimulation with tunicamycin is needed. As result, HERP expression is increased (Fig. 7) that means HERP expressions is regulated by ER stress response as known as UPR (Unfolded Protein Response) (Kokame K et al., 2000).

The actual mechanism of homocysteine-induced ER stress is still unknown. But, there some studies showed that a disulfide exchange reaction between the reactive thiol group of homocysteine and cysteine residues of ER proteins lead to misfolding (Lentz and Sadler.,1991), homocysteine decreases the extracellular super dismutase (EC-SOD) glycoprotein that protects the vascular wall from oxidative stress) mRNA expression and protein secretion (Perla., 2007), homocysteine (sulfhydryl group) reduces disulfide bonds of protein in the ER and causes accumulation of misfolded proteins in the ER (Zhang., 2001).

Also, the mechanism of ER stress-induced leptin resistance is still unclear. But, based on the molecular mechanisms of ER stress-induced leptin resistance, ER stress induces leptin resistance is mediated through PTP1B (protein tyrosine phosphatase 1B). Where, PTP1B significantly reversed ER stress-induced leptin resistance (Hosoi et al., 2008).

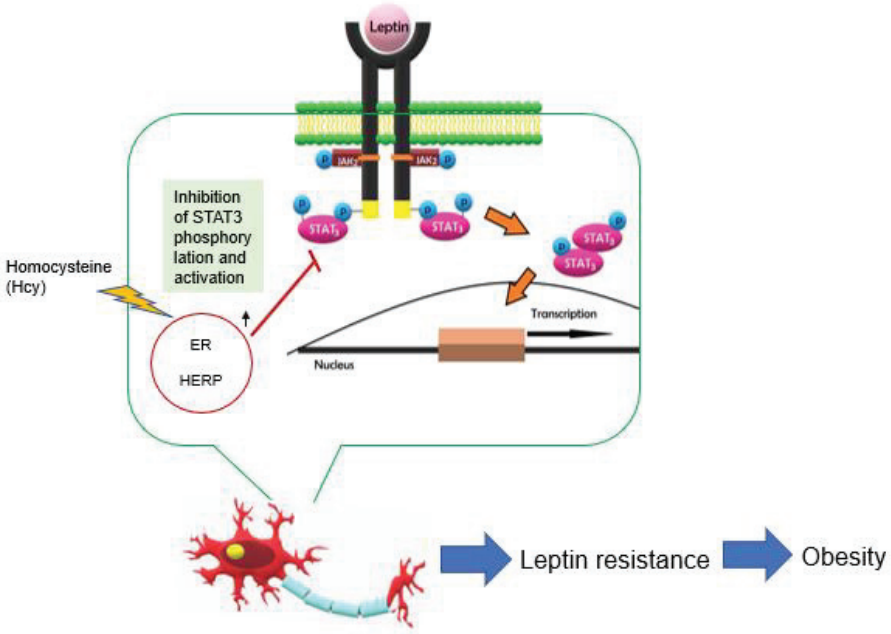
Other homocysteine-related compounds are methionine and cysteine, which have similar structural formulas. However, the results showed that only homocysteine strongly inhibited leptin signalling, whereas cysteine and methionine had no significant effect. Homocysteine and cysteine have thiols in their structural formula, whereas methionine has a sulfide. Therefore, it is possible that the thiol, not the sulfide, is involved in the development of leptin resistance. Another reason is according to the homocysteine metabolism, the reaction from SAH (S-adenosyl-homocysteine) to homocysteine is reversible.

High concentrations of homocysteine may increase the concentration of SAH and vice versa. While, methionine and cysteine are not.

Normal blood homocysteine levels range from 5 to 12 μM, and hyperhomocysteinemia is classified as mild (12-30 μM), moderate (30-100 μM), and severe (>100 μM) (Škovierová H et al., 2016; Ji C et al., 2004). While in the in vitro experiment, the used concentrations vary. They are 0,1 mM, 1 mM, 2 mM, 3 mM (Zhang X et al., 2021; 50. Deniz YILDIZ et al., 2010), 2.5 mM (Sato K et al., 2020), 5 mM (Lipton SA et al., 1997), and 10 mM (Althausen S and Paschen W., 2000). Also, we cannot exclude that local homocysteine concentrations around neurons may reach even higher values. High levels of homocysteine have been found in obese humans (Karatela RA and Sainani GS., 2009; Vayá A et al., 2012) and in obese mice (Yun KU et al., 2013). A study also revealed that an increase in total body fat percent and lower lean mass are associated with increased homocysteine concentrations (Al-Bayyari et al., 2017).

Because we would like to examine the leptin signal, we majorly focused on in vitro experiment, using SHSY5Y cell lines expressing Ob-Rb. In in vivo experiment, the Wistar of the National Institute of Nutrition obese (WNIN/Ob) seemed to be an appropriate animal model for obesity and other metabolic diseases. These rats are reported to show the characteristics of obesity such as insulin resistance and leptin resistance. They also are linked to decreased antioxidants which are then associated with aging problems (Sinha JK et al., 2014; Ghosh S et al., 2019). It would be an important future subject to analyse in vivo effects using these models.

In light of our results and the link between homocysteine and obesity, it is possible that homocysteine plays a key role in leptin resistance by inducing ER stress, contributing to the development of obesity and metabolic syndrome in conditions of hyperhomocysteinemia. Considering our results, we suggest that induction of ER stress by homocysteine could constitute one of the mechanisms of leptin resistance (Fig 8).




A high level of homocysteine would trigger stress in the ER, which could subsequently alter specifically the JAK2-STAT3 signaling pathway regulated by the hormone leptin. When ER stress occurs, the inhibition of STAT3 phosphorylation increases (Song M et al., 2020; Gan L et al., 2017; Kimura K et al., 2012; Hansen IS et al., 2019). ER stress activates three signaling networks, they are IRE1, PERK, and ATF6 Oslowski CM and Urano F., 2011). PERK is reported to be stimulated by JAK-STAT signaling (Wang L et al., 2015; Meares GP et al., 2014; Bao Y et al., 2021). The IRE1 signaling pathway is also affected by the JAK-STAT signaling where the inhibition of the IRE1 signaling reduces STAT signaling (Liang Y et al., 2020; Gonella R et al., 2021). Even the mechanisms are still unknown, but the alteration of XBP1 is reported to disrupt the phosphorylation of STAT3. IRE1/XBP1 and STAT3 signaling pathways are closely related (Argemí J et al., 2017).

There was no report of homocysteine on leptin resistance in neuronal cells. We found for the first time that homocysteine causes leptin resistance in neuronal cells. We also found that the effect of homocysteine on leptin signaling may be specific to leptin receptor signaling, as we did not observe the inhibition of STAT3 signaling against IL-6. Targeting the homocysteine metabolic pathway and/or inhibiting the excessive action of homocysteine could have therapeutic value.

# CHAPTER IV

# CONCLUSIONS

1. Homocysteine leads to significantly suppress of leptin receptor induced signaling pathway.
2. Homocysteine induces endoplasmic reticulum stress.
3. Homocysteine has no impact on the IL-6 signalling pathway.
4. Homocysteine has a toxicity effect that may cause cell death.
5. Methylation may indirectly be involved in the cause of leptin resistance.

Homocysteine may ameliorate the leptin signaling pathway by inhibiting JAK-STAT signaling that is regulated by the obesity hormone, leptin. In conclusion, homocysteine could cause neuronal leptin resistance by triggering endoplasmic reticulum stress, which would be one of the mechanisms of obesity.

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Basic article

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Preninka AI, Kuriya K, Yazawa K, Yoshii M, Yanase Y, Jockers R, Dam J, Hosoi T, Ozawa K. Homocysteine causes neuronal leptin resistance and endoplasmic reticulum stress. PLoS One. 2022 Dec 13;17(12):e0278965. doi: 10.1371/journal.pone.0278965. PMID: 36512575; PMCID: PMC9746958.