

Restraint-Induced Expression of Endoplasmic Reticulum Stress-Related Genes in the Mouse Brain

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ABSTRACT

Depression is a significant public health concern but its pathology remains unclear. Previously, increases in an endoplasmic reticulum (ER) stress-related protein were reported in the temporal cortex of subjects with major depressive disorder who had died by suicide. This finding suggests an association between depression and ER stress. The present study was designed to investigate whether acute stress could affect the ER stress response. Mice were immobilized for a period of 6 hr and then expression of ER stress response-related genes was measured by real-time PCR. We also used enzyme-linked immunosorbent assay for concomitant measurement of the plasma corticosterone levels in the mice. The effect of corticosterone on ER stress proteins was further investigated by treating mice with corticosterone for 2 weeks and then measuring ER protein expression by Western blotting. After a 6 hr restraint stress, mRNA levels of ER stress-related genes, such as the 78-kilodalton glucose regulated protein (GRP78), the 94-kilodalton glucose regulated protein (GRP94), and calreticulin, were increased in the cortex, hippocampus, and striatum of mouse brain. Blood plasma corticosterone level was also increased. In the corticosterone-treated mouse model, the expression of GRP78 and GRP94 was significantly increased in the hippocampus. These results suggest that acute stress may affect ER function and that ER stress may be involved in the pathogenesis of restraint stress, including the development of depression.

Keywords: Corticosterone, Depression, Endoplasmic Reticulum Stress, Restraint Stress

1. Introduction

Major depression, along with bipolar disorder, has become a common psychiatric disorder in modern society. About 1% of the population is estimated to be affected by major depression one or more times during their lifetime [1]. Even though extensive studies have led to a variety of hypotheses regarding the molecular mechanism underlying depression, the pathogenesis of this disorder remains to be fully elucidated.

The endoplasmic reticulum (ER) is the cell organelle where secretory and membrane proteins are synthesized and folded. It also functions as a Ca²⁺ store and resource of calcium signals. The disturbance of ER functions through events such as disruption of Ca²⁺ homeostasis, inhibition of protein glycosylation or disulfide bond formation, hypoxia and viral or bacterial infection, can result in the accumulation of unfolded or misfolded pro-

teins and may trigger stress responses in the cell (ER stress). To overcome ER stress, an unfolded protein response (UPR) is invoked by the activation of several signaling pathways; this UPR promotes an adaptive response to ER stress and reestablishes homeostasis in the ER [2,3]. Molecular chaperones such as the 78-kilodalton glucose regulated protein (GRP78) and the 94-kilodalton glucose regulated protein are induced and promote correct protein folding. If the damage is too severe to repair, C/EBP-homologous protein (CHOP) and other factors are activated and induce cell apoptosis [4]. On the other hand, if misfolded protein aggregates into insoluble higher-order structures, it can give rise to various diseases. For example, rhodopsin misfolding causes autosomal dominant retinitis pigmentosa [5], while the accumulation of amyloid β -peptide is associated with Alzheimer's disease [6].

Some reports have also suggested a relationship between mental disorder and ER stress. In bipolar disorder patients, DNA microarray analysis of cell derived from twins discordant with respect to the disease revealed a down-regulated expression of genes related to ER stress responses such as x-box binding protein 1 (XBP1) and GRP78 [7]. In schizophrenia patients, a similar abnormality of these genes was found [8]. In addition, mood-stabilizing drugs such as valproate and lithium have been reported to increase the expression of GRP78, GRP94, and calreticulin [9]. Similarly, olanzapine, one of the second-generation “atypical” anti-psychotic drugs, appears to potentiate neuronal survival and neural stem cell differentiation by regulation of ER stress response proteins [10].

A recent study reported that significantly increased levels of GRP78, GRP94, and calreticulin were found in the temporal cortex of subjects with major depressive disorder who had died by suicide compared with control subjects who had died of other causes [11]. In addition, hippocampal atrophy [12] and reduction of glial density in the subgenual prefrontal cortex [13] were found in patients with major depression. Stress, a risk factor for depression, has been shown to induce atrophy of the apical dendrites of the hippocampal neurons [14], and to promote neuronal apoptosis in the cerebral cortex [15] in animal depression models. These findings suggest that a stressful situation, which may increase the risk for suicide, serves as an ER stressor. To clarify the relationship between exogenous stress and ER stress, in the present study, we investigated the expression of ER stress-related genes after restraint stress. We also focused on the elevation of corticosterone in the plasma and used a corticosterone-treated depression model to clarify the relationship between chronic corticosterone elevation and ER stress.

2. Materials and Methods

2.1. Animals

Male 9-week-old ddY mice and male 6-week-old ICR mice (Japan SLC, Hamamatsu, Japan) were used for all experiments. Mice were housed at $24 \pm 2^\circ\text{C}$ under a 12 hr light-dark cycle (lights on from 8:00 to 20:00) and had ad libitum access to food and water when not under restraint. Animals were acclimatized to laboratory conditions before the experiment. All procedures relating to animal care and treatment conformed to the animal care guidelines of the Animal Experiment Committee of Gifu Pharmaceutical University. All efforts were made to minimize both suffering and the number of animal used.

2.2. Restraint Stress

Male 9-week-old ddY mice (Japan SLC) weighing 30-40

g were used for real-time PCR studies. Mice were placed into 50-mL perforated plastic tubes, which prevented them from turning in any direction. Each mouse was maintained in the tube for 6 hr without any access to food or water.

2.3. Sampling

After this restraint stress, a blood sample was collected from the tail and the mouse was decapitated. The brain was quickly removed from the skull, briefly washed in ice-cold saline, and laid on a cooled (4°C) metal plate. The brain was rapidly dissected to separate the hippocampus, striatum, and cortex and stored at -80°C until use.

2.4. RNA Isolation

Total RNA was isolated from frozen brain using High Pure RNA Isolation Kit (Roche, Tokyo, Japan). RNA concentrations were determined spectrophotometrically at 260 nm. First-stranded cDNA was synthesized in a 20- μl reaction volume using a random primer (Takara, Shiga, Japan) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

2.5. Real-Time PCR

Real-time PCR (TaqMan; Applied Biosystems, Foster City, CA, USA) was performed as described previously [16]. Single-standard cDNA was synthesized from total RNA using a high capacity cDNA archive kit (Applied Biosystems). Quantitative real-time PCR was performed using a sequence detection system (ABI PRISM 7900HT; Applied Biosystems) with a PCR master mix (TaqMan Universal PCR Master Mix; Applied Biosystems), according to the manufacturer's protocol. A gene expression product (Assays-on-Demand Gene Expression Product; Applied Biosystems) was used for measurements of mRNA expression by real-time PCR. The primers used for amplification were as follows: GRP78: 5'-GTTTGCTGAGGAAGACAAAAAGCTC-3' and 5'-CACTTCCATAGAGTTTGCTGATAATTG-3'; CHOP: 5'-GGAGCTGGAAGCCTGGTATGAGG-3' and 5'-TCCCTGGTCAGGCGCTCGATTTC-3'; GRP94: 5'-CTCACCATTGGATCCTGTGTG-3' and 5'-CACATGACAAGATTACATCAAGA-3'; calreticulin: 5'-GCCAAGGACGAGCTGTAGAGAG-3' and 5'-GGTGAGGGCTGAAGGAGAATC-3'; ERdj4: 5'-TCTAGAATGGCTACTCCCAGTCAATTTTC-3' and 5'-TCTAGACTACTGTCCGAACAGTCAGTG-3'; EDEM: 5'-TGGGTTGGAAAGCAGAGTGGC-3' and 5'-TCCATTCCTACATGGAGGTAG-3'; p58IPK 5'-GAGGTTTGTGTTTGGGATGCAG-3' and 5'-GCTCTTCAGCTGACTCAATCAG-3'; ASNS: 5'-AGGTTGATGATGCAATGATGG-3' and 5'-TCCCCTATCTACCCACAGTCC-3'; β -actin: 5'-TCCTCCCT

GGAGAAGAGCTAC-3' and 5'-TCCTGCTTGCTGATCCACAT-3' The thermal cycler conditions were as follows: 2 min at 50°C and then 10 min at 95°C, followed by two-step PCR for 50 cycles consisting of 95°C for 15s followed by 60°C for 1 min. For each PCR measurement, we checked the slope value, R² value, and linear range of a standard curve of serial dilutions. All reactions were performed in duplicate. The results were expressed relative to a β -actin internal control.

2.6. Measurement of Plasma Corticosterone

Plasma was obtained as described previously [17] and the concentration of corticosterone was determined *via* a corticosterone EIA kit (Assay Designs, Inc., Ann Arbor, MI, USA) according to the manufacturer's protocol.

2.7. Chronic Corticosterone Treatment

Male 6-week-old ICR mice (Japan SLC) weighing 20-25 g were used for chronic oral corticosterone exposure as described in a previous report [18]. Briefly, corticosterone (25 μ g/mL free base; 4-pregnen-11 β 21-DIOL-3 20-DIONE 21-hemisuccinate; Steraloids, Inc., RI, USA) was added to tap water and the pH was brought to 12-13 with 10 N NaOH (Kishidai Chemical, Osaka, Japan), followed by stirring at 4°C until dissolved (3 to 7 hr). Following dissolution, the pH was brought to 7.0-7.4 with 10 N HCl (Wako, Osaka, Japan). Group-housed ICR mice were presented with this corticosterone solution in place of normal drinking water for 14 days, resulting in a dose of approximately 8.7 mg/kg/day (p.o). Animals were weaned with 3 days of 12.5 μ g/mL, and then 3 days with 6.25 μ g/mL, to allow for gradual recovery of endogenous corticosterone secretion.

2.8. Western Blot Analysis

At 35 days, each mouse was decapitated and its brain was quickly removed from the skull, briefly washed in ice-cold saline, and laid on a cooled (4°C) metal plate. The brain was rapidly dissected to separate the hippocampus and stored at -80°C until use. Brain samples were homogenized in 10 mL/g tissue ice-cold lysis buffer [50 mM Tris-HCl (pH 8.0) containing 159 mM NaCl, 50 mM EDTA, 1% Triton X-100, and protease/phosphatase inhibitor mixture] using a homogenizer (Phycotron; Microtec Co. Ltd., Chiba, Japan). Lysates were centrifuged at 12,000 \times g for 15 min at 4°C. Supernatants were collected and boiled for 5 min in SDS sample buffer (Wako). Equal amounts of protein were subjected to 10% SDS-PAGE gradient gel and then transferred to poly(vinylidene difluoride) membranes (Immobilon-P; Millipore, MA, USA). After blocking with Block Ace (Snow Brand Milk Products Co. Ltd., Tokyo, Japan) for 30 min, the membranes were incubated with primary antibody. The

primary antibodies used were as follows: mouse anti-BiP antibody (BD Bioscience, CA, USA) for GRP78, mouse anti-KDEL antibody (Stressgen Bioreagents Limited Partnership, B.C., Canada) for GRP94, and mouse anti-actin antibody (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, the membrane was incubated with the secondary antibody [goat anti-mouse (Pierce Biotechnology, IL, USA)]. The immunoreactive bands were visualized using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and then measured using LAS-4000 mini (Fujifilm, Tokyo, Japan).

2.9. Statistical Analysis

Statistical comparisons were made by Student's *t*-test using Statview version 5.0 (SAS Institute Inc., NC, USA), with $p < 0.05$ being considered statistically significant.

3. Results and Discussion

Real-time PCR was carried out to investigate whether the expression of ER stress response-related genes in the brain was changed by 6-hr restraint stress. In this study, we investigated the expression of GRP94, calreticulin, ER degradation-enhancing α -mannosidase-like protein (EDEM), protein kinase inhibitor of 58 kDa (p58^{IPK}), asparagine synthetase (ASNS), GRP78, ER-localized DnaJ 4 (ERdj4), and C/EBP homologous protein (CHOP). The expression of GRP78, GRP94, and calreticulin mRNA was significantly increased in the hippocampus, striatum, and cortex (**Figure 1**). In addition, there was significantly increased expression of p58^{IPK} mRNA in the cortex, but not in the hippocampus or striatum.

We next investigated whether restraint stress affected the plasma concentrations of corticosterone, as previously reported. Immediately following the 6-hr restraint stress, significantly higher plasma corticosterone concentrations were found in stressed mice compared to unstressed mice. Seven days after the restraint stress, the plasma corticosterone recovered to the normal control level (**Figure 2**).

To clarify the mechanism of ER stress-related mRNA elevation, we artificially elevated the plasma concentrations of corticosterone in mice for 2 weeks and then measured the levels of ER stress-related proteins. In the corticosterone-treated animal model, the expression of GRP78 and GRP94 in the hippocampus was significantly increased compared to control levels (**Figure 3**).

Restraint stress is used widely to induce stress responses in animals, and it is known that a number of stresses, including restraint stress, can cause depression in animals. In the present study, we found that several ER stress-related genes were increased in the mouse hippocampus, striatum, and cortex after restraint stress.

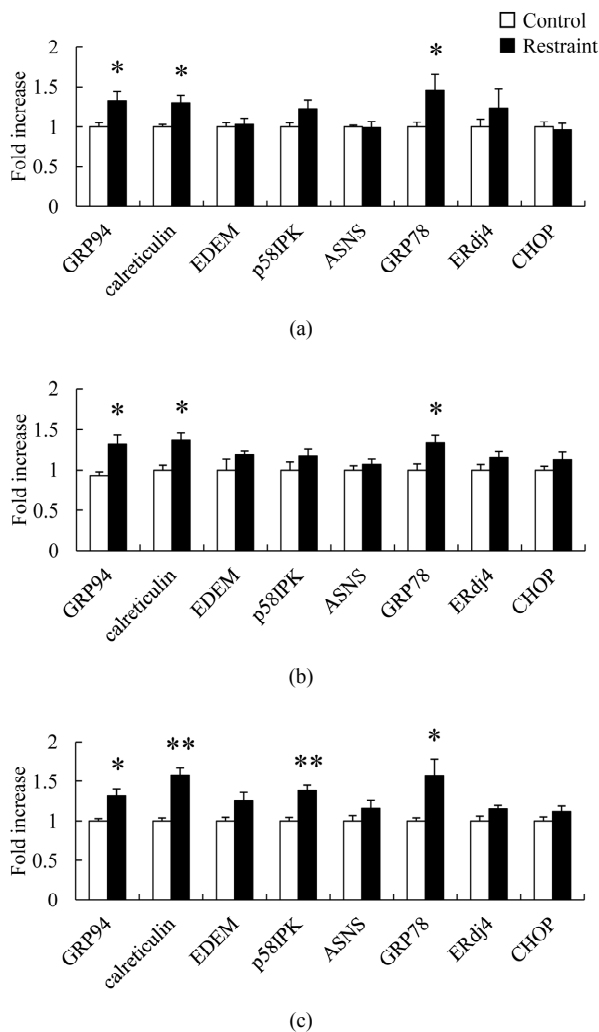


Figure 1. The expression mRNA of ER stress-related factors in the mouse brain after 6 hr restraint-stress. Mice were immobilized for 6 hr in a 50-mL perforated plastic tube. White and black bars represent the control group and the restraint group, respectively. Immediately after restraint, mice were killed and real-time PCR was performed on brain tissues from the (a) hippocampus, (b) striatum, and (c) cortex. Data represent means and S.E.M., n = 3 to 5. *p < 0.05, **p < 0.01 vs. control group. GRP94: the 94-kilodalton glucose regulated protein, EDEM: ER degradation-enhancing α -mannosidase-like protein, p58IPK: protein kinase inhibitor of 58 kilodalton, ASNS: asparagines synthetase, GRP78: the 78-kilodalton glucose regulated protein, ERdj4: ER-localized DnaJ 4, CHOP: C/EBP-homologous protein.

The significant increases in expression of GRP78, GRP94, and calreticulin agreed with the findings of a previous report of changes in the temporal cortex of subjects with major depression who died by suicide [11]. However, no study has yet specifically investigated expression changes of these genes in the hippocampus or the striatum in subjects with depression.

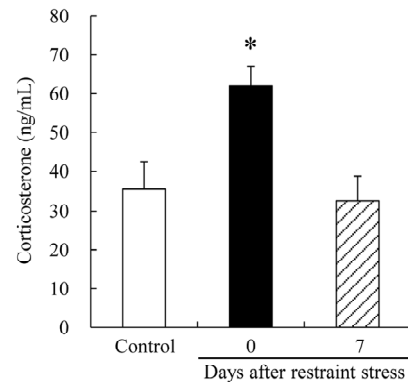


Figure 2. The effect of 6 hr restraint stress on the concentration of corticosterone in mouse plasma. Mice were immobilized for 6 hr. Immediately after restraint and 7 days later, blood samples were collected and concentration of plasma corticosterone was measured by ELISA. Restraint stress significantly increased the concentration of corticosterone in plasma. The corticosterone levels decreased to the normal control levels 7 days after restraint stress. Data represent means and S.E.M., n = 7. *p < 0.05 vs. control group.

GRP78, otherwise known as BiP, is one of the best-characterized ER chaperone proteins and is regarded as a classical marker of UPR activation. Overexpression of GRP78 has been reported to inhibit the upregulation of CHOP, which plays a key role in regulating cell growth and which has been implicated in apoptosis [19,20]. GRP94 and calreticulin are also ER chaperone proteins and show protective effects against ER stress [21]. The increase in these chaperones after restraint stress (Figure 1) may represent an attempt to oppose the toxic effect of prolonged stress and the high concentrations of glucocorticoid, such as corticosterone, on the brain. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, which controls glucocorticoid levels, has been reported in most depression patients and glucocorticoid level of depression patients was higher than those of normal ones [22-24]. In the mice in the present study, 6-hr restraint stress elevated the concentration of corticosterone in plasma, suggesting that restraint stress induced a response similar to depression.

Recently, corticosterone has been reported to exert immunostimulatory effects on macrophages *via* induction of ER stress [25]. Following corticosterone treatment, the glucocorticoid receptor (GR) binds onto B-cell lymphoma 2 (Bcl-2), a protein that affects cytochrome C and calcium release from mitochondria. Subsequently, this GR/Bcl-2 complex moves into mitochondria and regulates mitochondrial functions in an inverted “U”-shaped manner—i.e., a high dose treatment with corticosterone decreased levels of GRs and Bcl-2 in mitochondria and intracellular calcium was increased [26,27]. Substances

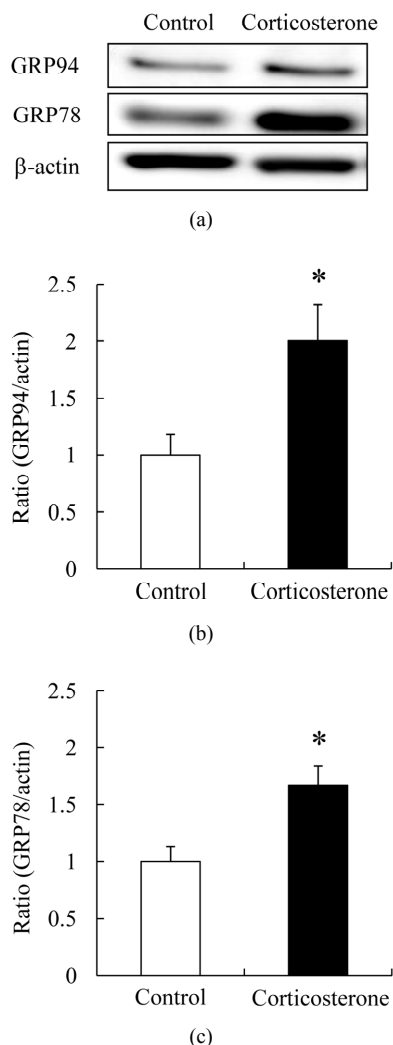


Figure 3. The expression of GRP78 and GRP94 in the hippocampus in a mouse model of chronic corticosterone induced depression. (a) Representative band images show immunoreactivities against GRP94, GRP78, and β -actin. (b) GRP78 expression was significantly increased by corticosterone exposure. (c) GRP94 expression was also increased by corticosterone exposure. Data represent means and S.E.M., $n = 5$ or 6 . * $p < 0.05$ vs. control group.

that deplete the ER Ca^{2+} stores, such as thapsigargin, are widely used as ER stressors. Therefore, elevation of Ca^{2+} via GR may be sufficient for control of ER stress responses. In the present study, the restraint stress induced the expressions of only GRP78, GRP94, and calreticulin, but not other ER proteins. GRP78, GRP94, and calreticulin function as Ca^{2+} binding proteins [28]. Under the high concentration of corticosterone, the intracellular Ca^{2+} level might be higher, therefore, the expressions of GRP78, GRP94, and calreticulin might be increased.

Intracerebroventricular administration of thapsigargin has been reported to produce a depressant-like behavior

[29]. A 14-days corticosterone treatment has also shown to induce depression symptoms in mice [18]. We used this animal model to investigate the effect of chronic elevation of corticosterone on ER stress responses in brain. As expected, significant increases in GRP78 and GRP94 proteins were observed in the hippocampus (Figure 3). The increase of GRP78 was consistent with the result of a previous report [30]. On the other hand, no change in these proteins was observed in the cortex (data not shown). Mineralocorticoid receptor (MR) and GR, which are the targets of corticosterone, are known to be well expressed in the hippocampus [31,32]. These reports, together with our findings, indicate that the hippocampus may be more sensitive to corticosterone exposure than are other brain regions. Many reports have referred to hippocampal atrophy in patients with depression [12,14]. In the cortex, it had been reported that chronic stress increased the caspase-3 positive neurons, in other words, exogenous stress was contributing to the cell apoptosis [15]. In our study, corticosterone exposure was performed for 2 weeks, but, in fact, long-term cortisol elevation has been observed in most depression patients. More extended corticosterone treatment may affect the expression of ER stress proteins in the cortex.

Recently, many experiments have focused on the relationship between depression and neurogenesis. Interestingly, ER stress also affects adult neurogenesis in the brain [33]. Brain-derived neurotrophic factor (BDNF), which promotes neurogenesis, is also known to inhibit neuronal cell death induced by ER stress [34]. These reports may also point to an involvement of ER stress in depression.

4. Conclusions

Restraint stress, which may contribute to depression in mice, may up-regulate the ER stress response via corticosterone elevation. This suggests the possibility of an ER stress involvement in the pathogenesis of stress-related depression disorders.

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