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A sigma-1 receptor antagonist (NE-100) prevents tunicamycin-induced cell death via GRP78 induction in hippocampal cells

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ABSTRACT

Endoplasmic reticulum (ER) stress is involved in various diseases such as ischemia, Alzheimer's disease, and Parkinson's disease. The widely used selective sigma-1 receptor antagonist, *N*, *N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride (NE-100), has been shown to suppress ischemia-induced neuronal cell death in the murine hippocampus. In the present study, we investigated whether NE-100 might suppress neuronal cell death that is induced by ER stress in ischemic injury. These studies show that NE-100 protected the ER stress-induced cell death of murine hippocampal HT22 cells, but not the oxidative stress-induced cell death. This suggests that NE-100 may have a protective effect on the ER. However, another sigma-1 receptor antagonist (BD1047) did not suppress ER stress-induced cell death. In addition, NE-100 attenuated the upregulation of C/EBP homologous protein (CHOP) induced by ER stress and upregulated the expression of both the 50-kDa activating transcription factor 6 (p50ATF6) and the 78-kDa glucose-regulated protein (GRP78). However, NE-100 did not impact the expression of phosphorylated eukaryotic initiation factor 2α (p-eIF2α) nor splicing of X-box-binding protein 1 (XBP-1). These findings suggest that NE-100 suppresses ER stress-induced cell death via CHOP expression by the upregulation of GRP78 through ATF6 pathway, independent sigma-1 receptor antagonist effect.

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1. Introduction

Endoplasmic reticulum (ER) stress is triggered by the accumulation of unfolded proteins due to dysfunction in the protein-folding capacity of ER and can lead to cellular damage [1]. Under ER stress, the self-protective unfolded protein response (UPR) activates to maintain protein homeostasis and promote cell survival [2]. The UPR activates three signaling proteins: 1) the protein kinase RNA -like ER kinase (PERK), 2) the activating transcription factor 6 (ATF6), and 3) the inositol-requiring enzyme 1 (IRE1). Activation of these three proteins occurs by their dissociation from the glucose-regulated protein 78 (GRP78)/immunoglobulin heavy-chain binding protein (BiP) [3–5]. GRP78 is a molecular chaperone with antiapoptotic properties and works to normalize the folding of the unfolded proteins during ER stress [6]. GRP78 is upregulated by the activation of the UPR and acts to restore the normal function of the ER [7]. However, if ER stress is prolonged and the UPR fails to recover ER function, the UPR induces transcription of CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP) to initiate apoptotic signaling pathways [8].

Ischemia-induced ER stress has been shown in numerous studies to induce cell death within the brain [9–11]. *N*, *N*-Dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl] -ethylamine monohydrochloride (NE-100) is a widely used selective sigma-1 receptor antagonist [12]. A previous study demonstrated that NE-100 suppressed ischemia-induced neuronal cell death in the hippocampus [13]. Therefore, in the present study we investigated the impact of NE-100 on ER stress-induced cell death in murine hippocampal HT22 cells and the underlying mechanism of action with respect to the UPR.

2. Materials and methods

2.1. Cell cultures

Murine hippocampal HT22 cells were a generous gift from Dr. Yoko Hirata (Gifu University, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and 100 μg/ml of streptomycin (Meiji Seika) in a humidified atmosphere containing 5% CO_2 at 37 °C. Since this cell line is adherent, cells were passaged with trypsin (source of trypsin) every 2–3 days as necessary to prevent confluency, and maintained in a 10-cm petri dish (BD Biosciences, Franklin Lakes, NJ, USA).

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2.2. Cell death assay

HT22 cells were plated at 3×10^3 cells per well in 96-well plates (BD Biosciences) and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. To investigate the actions of NE-100, three treatment groups with differing components were prepared: (1) vehicle containing 1% FBS and NE-100 (Santa Cruz Biotechnology, Inc., CA, USA; concentration, 0.1–3 μM), (2) vehicle containing 1% FBS and BD1047, another sigma-1 receptor antagonist (Santa Cruz; 0.1–10 μM) and (3) vehicle alone as a control. Cells were pretreated with one of these solutions for 1 h, followed by the addition of tunicamycin (Wako Pure Chemical Industries, Ltd. Osaka, Japan; 50 ng/ml), glutamate (3 mM), or vehicle for 24 h. NE-100, tunicamycin, and glutamate were dissolved in phosphate-buffered saline (PBS; pH 7.4) containing 1% dimethyl sulfoxide (DMSO) as vehicle. Tunicamycin inhibits glycosylation of newly synthesized proteins and induces ER stress [14]. High concentrations of extracellular glutamate inhibit cystine uptake through a glutathione/cystine antiporter and induced oxidative stress in HT22 cells [15,16]. Because cystine is a precursor for glutathione (GSH) synthesis, cellular GSH levels decline and reactive oxygen species (ROS) accumulate and induce oxidative stress in HT22 cells [17]. Cell death was detected using dual staining with Hoechst 33342 (Invitrogen, CA, USA) and propidium iodide (PI; Invitrogen). Hoechst 33342 stains all cells (living and dead cells), whereas PI staining measures late apoptotic and necrotic cells [18]. Therefore, in the present study, tunicamycin-induced cell death represented the late apoptosis and necrosis, but not the early apoptosis. In addition, nuclear staining was also done after the 24-h incubation with tunicamycin or glutamate. At the end of the culture period, the Hoechst 33342 (λ_{ex} = 360 nm, λ_{em} > 490 nm) and PI (λ_{ex} = 535 nm, λ_{em} > 617 nm; Molecular Probes) dyes were added to the culture medium (8 and 1.5 μM, respectively) for 15 min [19]. Images were collected with an inverted epifluorescence microscope (IX70; Olympus. Co., Tokyo, Japan) and a CCD camera (DP30BW; Olympus). A blinded observer counted the number of cells per condition with the aid of image-processing software (Image-J, version 1.33f; National Institutes of Health, Bethesda, MD, USA). Cell mortality was quantified by expressing the number of PI-positive cells as a percentage of the number of Hoechst 33342-positive cells.

2.3. Western blot analysis

HT22 cells were plated at 3×10^4 cells per well in 12-well plates (BD Biosciences) and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. To investigate the actions of NE-100 against various proteins, two treatment groups with differing components were prepared: (1) vehicle containing 1% FBS and NE-100 (Santa Cruz, 3 μM) and (2) vehicle alone as a control. Cells were pretreated with one of these solutions for 1 h, followed by the addition of tunicamycin (Wako Pure Chemical Industries, Ltd. Osaka, Japan; 50 ng/ml) or vehicle for 24 h. NE-100 and tunicamycin were dissolved in phosphate-buffered saline (PBS; pH 7.4) containing 1% dimethyl sulfoxide (DMSO) as vehicle. At the end of the culture period, HT22 cells were washed with PBS twice in order to remove dead cells. The HT22 cells were then lysed using a cell lysis buffer (RIPA buffer: 50 mM Tris HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1% Igepal CA-630) together with protease inhibitors (Sigma–Aldrich, St. Louis, MO, USA) and a phosphatase inhibitor cocktail (Sigma–Aldrich). The lysate was centrifuged and the supernatant was collected. Protein concentration was determined from a standard curve of bovine serum albumin (BSA) with a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). A mixture of equal parts of a protein sample and sample buffer with 20% 2-mercaptoethanol (Wako) was subjected to 5–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Wako). The separated protein was then transferred onto a polyvinylidene difluoride membrane (PVDF, Immobilon-P; Millipore Corporation, Bedford, MA, USA). To visualize the proteins on the membrane, the following primary antibodies were used: mouse anti-GRP78 antibody (Santa Cruz), mouse anti-CHOP (GADD153) antibody (Santa Cruz), rabbit anti-ATF6 antibody (Abcam, Cambridge, UK), rabbit phospho-eIF2α antibody (Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit eIF2α antibody (Cell Signaling Technology, Inc.), and mouse anti-β-actin monoclonal antibody (Sigma–Aldrich). The secondary antibodies were goat anti-mouse HARP-conjugated and goat anti-rabbit HARP-conjugated (Thermo Fisher Scientific Inc.). The immunoreactive bands were visualized using a chemiluminescent substrate (ImmunoStar® LD; Wako). The band intensity was measured using an imaging analyzer (LAS-4000; Fuji Film, Tokyo, Japan).

2.4. Real time-PCR

Total RNA was isolated from HT22 cells according to the manufacturer's protocol for NucleoSpin® RNA II (MACHEREY–NAGEL GmbH & Co., KG, Germany). First-strand cDNA was synthesized from total RNA in a 20-μL reaction mixture by using a PrimeScript® RT reagent Kit (Takara BIO INC, Shiga, Japan). Real-time PCR was performed in Thermal Cycler Dice Real Time System II (Takara) using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; Takara). PCR amplication had a 30-s denaturing step at 95 °C, followed by two-step PCR comprising 5 s at 95 °C and 30 s at 60 °C, with 40 cycles for XBP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For XBP-1, the forward primer was 5′-GAACCAGGAGTTAAGAACACG-3′ and the reverse primer was 5′-AGGCAACAGTGTCAGAGTCC-3′. For GAPDH, the forward primer was 5′-TGTGTCC GTCGTGGATCTGA-3′ and the reverse primer was 5′-TTGCTGT TGAAGTCGCAGGAG-3′. Quantitative real-time PCR analysis was performed with a Thermal Cycler Dice™ Real Time System TP 800 (Takara).

2.5. Statistical analysis

Data are presented as the mean ± standard error of the mean (S.E.M.). Statistical comparisons were made using a two-tailed paired Student's *t*-test, Welch's *t*-test, or one-way ANOVA, followed by Dunnett's test, where $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. NE-100 protected against murine hippocampal HT22 cell death induced by ER stress, but not by oxidative stress

Cell death assay was first used to investigate the effect of NE-100 against tunicamycin-induced cell death in murine hippocampal HT22 neuronal cells. NE-100 at 0.1–3 μM protected HT22 cells against tunicamycin-induced cell death in a concentration-dependent manner, the effect being significant at 0.3–3 μM (Fig. 1A and B). On the other hand, NE-100 did not affect glutamate-induced cell death (Fig. 1C and D). In addition, BD1047, a sigma-1 receptor antagonist, did not protect HT22 cells against tunicamycin-induced cell death. Neither NE-100 nor BD1047 alone had an effect on cell viability (data not shown). These results demonstrate that NE-100 is capable of specifically protecting the hippocampal cells from ER stress. Since maximum protective effect of NE-100 was observed at 3 μM, this concentration was used for all further studies.

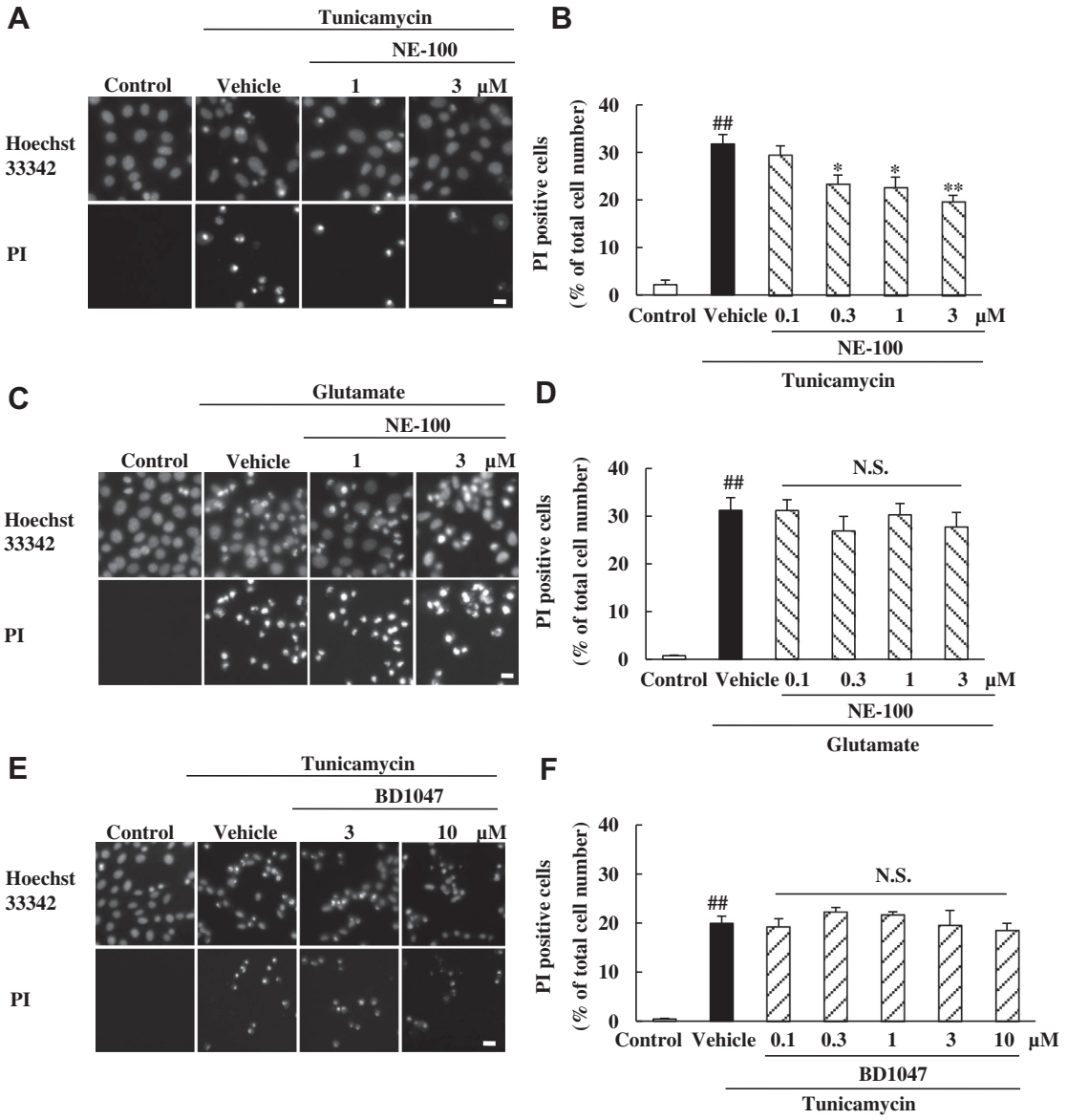

Fig. 1. NE-100 protected murine hippocampal HT22 cells against ER stress-induced cell death. (A, C, and E) Representative fluorescence images show nuclear staining for Hoechst 33342 (top images) and propidium iodide (PI, bottom images) in HT22 cells. Cells were pretreated with vehicle or with various concentrations of NE-100 or BD for 1 h, followed by tunicamycin (50 ng/ml) or glutamate (3 mM) for 24 h. (B, D, and F) The number of PI-positive cells and Hoechst 33342-positive cells were counted, and cell death was expressed as the percentage of PI-positive cells to Hoechst 33342-positive cells. $^{\#\#}P < 0.01$ versus control (Student's *t*-test). $^{*}P < 0.05$; $^{**}P < 0.01$ versus tunicamycin-treated (vehicle) group (Dunnett's test). Each column and bar represent mean ± S.E.M. ($n = 6–11$). N.S.: not significant. Scale bar = 50 μm.

3.2. NE-100 downregulated expression of CHOP, but not GRP78, after tunicamycin treatment and NE-100 alone induced GRP78 protein without CHOP protein induction in HT22 cells

We then investigated the effect of NE-100 on ER stress-related proteins such as GRP78 and CHOP. Tunicamycin treatment for 24 h significantly upregulated protein expression of both GRP78 and CHOP versus the control in HT22 cells (Fig. 2A and B). The expression level of GRP78 increased slightly at 24 h after NE-100 treatment alone, but this was not statistically significant (Fig. 2A). However, NE-100 significantly suppressed the expression of CHOP induced by tunicamycin (Fig. 2B). This demonstrated that NE-100 differentially affected protein expression after ER stress induced by tunicamycin. In addition, to clarify whether NE-100 treatment alone induced the GRP78 expression, we investigated the time-dependent expression level of GRP78 in HT22 cells treated with NE-100. GRP78 increased after NE-100 treatment in time-dependent manner, peaking at 12 h after treatment (Fig. 2C). At 12 h after treatment of NE-100, the expression of GRP78 was significantly increased compared to the vehicle group (Fig. 2D). Moreover, NE-100 treatment alone did not induce CHOP protein at any time, although tunicamycin induced CHOP protein at 12 h (Fig. 2E).

3.3. Induction of GRP78 expression by NE-100 is mediated by the ATF6 pathway

We then examined whether three major transducers of the ER stress response, namely, PERK, IRE, and ATF6, affect the induction of GRP78 by NE-100. NE-100 significantly upregulated expression of the 50-kDa ATF6 (p50 ATF6) at 6 h after treatment (Fig. 3A and B), but did not affect the expression of the 90-kDa ATF6 (p90 ATF6) (Fig. 3A and C). NE-100 did not promote phosphorylation of the eukaryotic initiation factor 2α (p-eIF2α) (Fig. 3D), which is

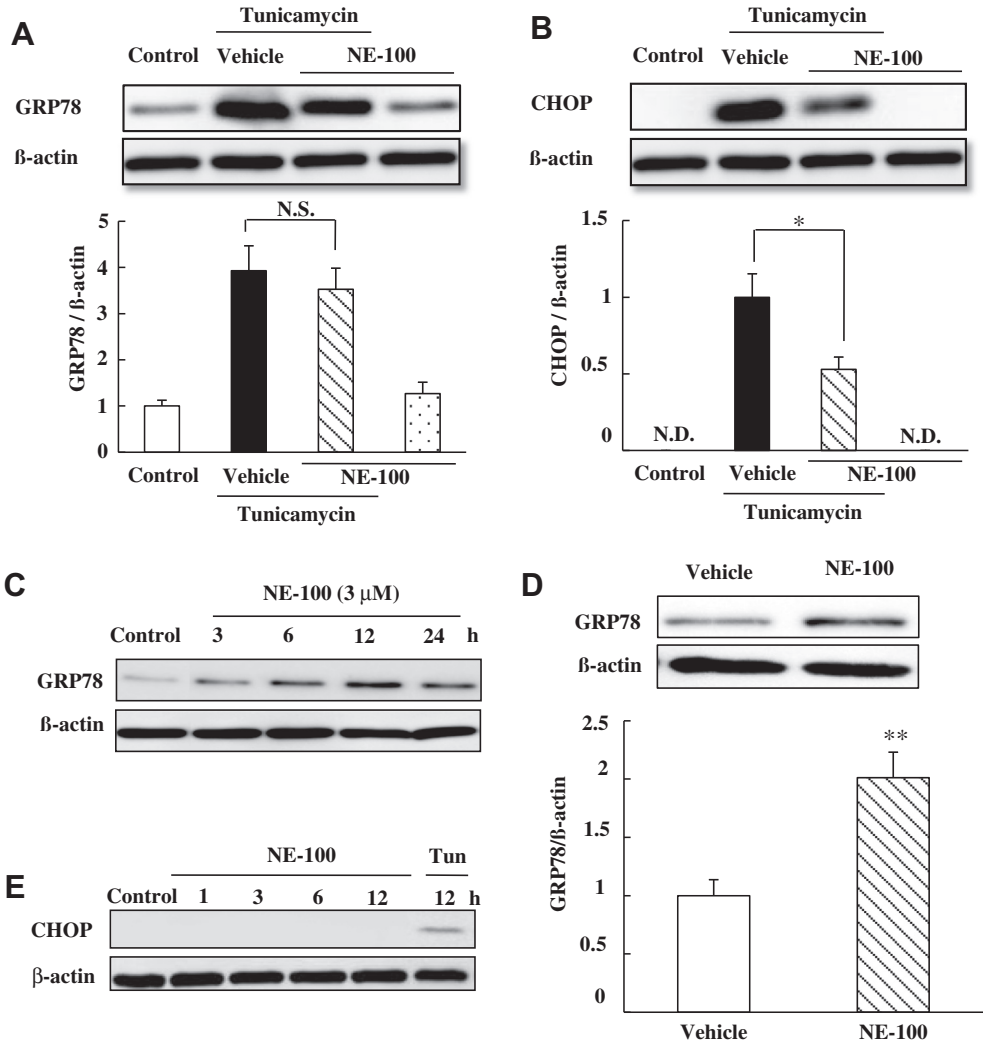

Fig. 2. NE-100 regulated expression of GRP78 and CHOP under ER stress conditions and NE-100 alone induced GRP78 protein without CHOP protein induction in HT22 cells. HT22 cells were pretreated with vehicle or with NE-100 (3 μM) for 1 h, and then treated with tunicamycin or vehicle for 24 h. Proteins were analyzed by western blot using antibodies specific to (A) GRP78 and (B) CHOP. Protein levels were quantified by densitometry and normalized to the level of β-actin. (C) HT22 cells were treated with NE-100 (3 μM) for 24 h, and GRP78 proteins were analyzed by western blot analysis at 3, 6, 12, and 24 h. (D) HT22 cell were treated with vehicle (as a control) and NE-100 (3 μM) for 12 h. GRP78 proteins were analyzed by western blot at 12 h. (E) HT22 cells were treated with NE-100 (3 μM) and CHOP proteins were analyzed by western blot at 1, 3, 6, and 12 h, and the cells were also treated with tunicamycin and CHOP proteins were analyzed at 12 h. $^{\#\#}P < 0.01$ versus control (Student's *t*-test). $^{*}P < 0.05$, $^{**}P < 0.01$ versus tunicamycin-treated (vehicle) group (Student's *t*-test). Each column and bar represent mean ± S.E.M. (n = 5–9), N.S.: not significant, N.D.: not determined, Tun: tunicamycin.

downstream of PERK. Furthermore, RT-PCR analysis was then performed for RNA isolates from tunicamycin-treated or NE-100-treated HT22 cells. XBP-1, a downstream of IRE1, mRNA was spliced under ER stress condition induced by tunicamycin, but the mRNA was not spliced by NE-100 alone (Fig. 3E).

4. Discussion

The purpose of this study was to investigate the effects of NE-100 against ER stress-induced neuronal cell death. Here, we demonstrated that NE-100 protected HT22 hippocampal cells against ER stress-induced cell death, while another sigma-1 receptor antagonist BD1047 did not demonstrate the same protective effect (Fig. 1). Hirata et al. showed that NE-100 was not involved in protecting cell viability against oxidative stress in HT22 cells [20]. This is supported by our studies here showing that NE-100 did not protect against oxidative stress-induced cell death (Fig. 1). These data indicate that the effect of NE-100 against ER stress is independent of the sigma-1 receptor.

NE-100 suppressed the upregulation of CHOP induced by ER stress (Fig. 2). Increased expression of CHOP triggers ER stress-induced cell death and CHOP expression is upregulated in various neurodegenerative diseases in animal models, such as ischemia, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) [10,21,22]. These studies suggest that NE-100 improves ER function, thereby suppressing ER stress-induced cell death. GRP78 assists in the folding of newly synthesized proteins and prevents aggregation of unfolded proteins [23,24]. GRP78 has also been showed to directly interact with the apoptotic pathway by blocking caspase activation, reducing CHOP expression, and ultimately inhibiting cell death and increasing cell survival [25–27]. Here, we show that NE-100 induced GRP78 expression where peak expression was observed at 12 h after treatment with NE-100 alone (Fig. 2). Taken together, these data suggest that NE-100 may induce GRP78, thus leading to attenuation of CHOP expression. NE-100 treatment led to an increase in p50 ATF6, but XBP-1 mRNA splicing and the expression of phosphorylated eIF2α (p-eIF2α) was not affected by the treatment of NE-100 (Fig. 3). Expression of GRP78 is induced by p-eIF2α and represses translation

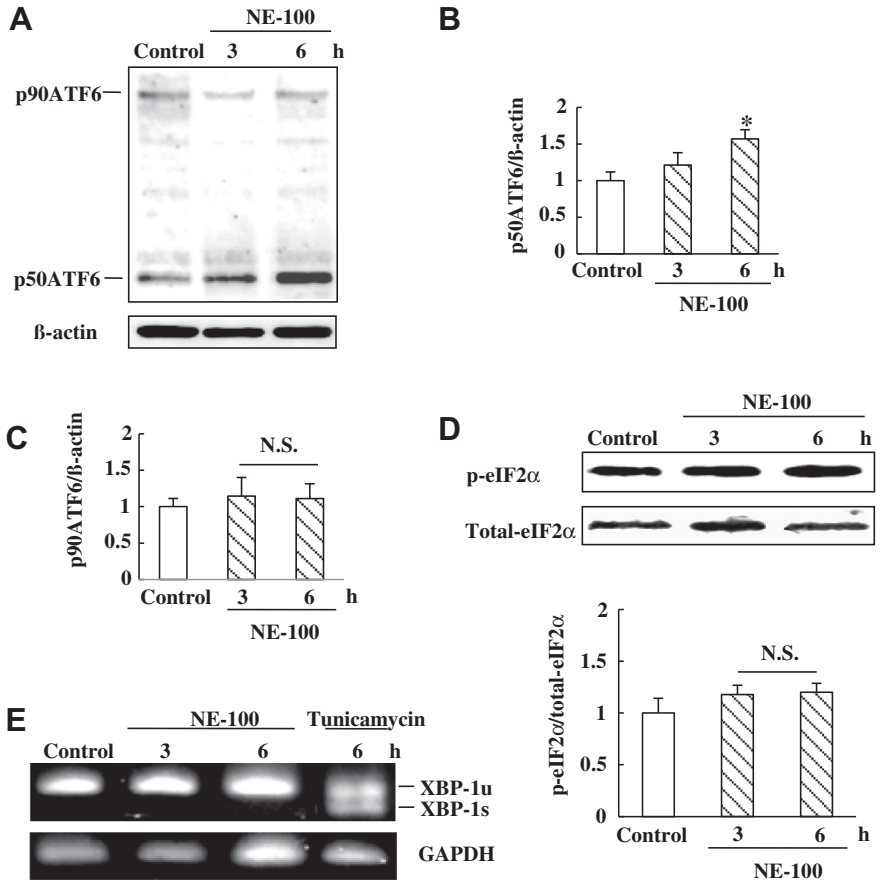

Fig. 3. Induction of GRP78 by NE-100 is mediated by the ATF6 pathway. (A) HT22 cells were treated with NE-100 (3 μM) for 6 h. The p90 ATF6 and p50 ATF6 were analyzed by western blot at 3 and 6 h. (B and C) Quantitative analysis of p50ATF6 and p90ATF6 protein levels. $^{*}P < 0.05$ versus control group (Dunnett's test). (D) HT22 cells were treated with NE-100 (3 μM) for 6 h. Phosphorylated-eIF2α (p-eIF2α) proteins were analyzed by western blot analysis at 3 and 6 h. (E) HT22 cells were treated with NE-100 (3 μM) or tunicamycin over 6 h. XBP-1 mRNA splicing was analyzed by PCR at 3 and 6 h. XBP-1u is unspliced XBP-1 and XBP-1s is spliced XBP-1. Each column and bar represent mean ± S.E.M. ($n = 4–6$). N.S.: not significant.

under stress [3,28]. The transcription factor p90 ATF6 is activated by posttranslational modifications. Under normal conditions, p90 ATF6 is transferred to the Golgi apparatus and cleaved by site-1 protease (S1P) and site-2 protease (S2P) [29]. After cleavage, the p50 ATF6 is released, which subsequently activates nuclear transcription of chaperones such as GRP78 [30]. IRE1 autophosphorylates and activates its endoribonuclease activity, causing the XBP-1 mRNA to splice [31]. The spliced XBP-1 then functions as a transcriptional transactivator of GRP78 in the nucleus [32]. Here, we show that NE-100 upregulated expression of p50 ATF6 (Fig. 3). Therefore, these results suggest that NE-100 may induce the GRP78 expression via the ATF6 pathway, not via PERK or IRE1 pathways. In our previous studies, we showed that BiP inducer X (BIX) induced GRP78 through the ATF6 pathway and protected neuronal cells against brain ischemia and tunicamycin-induced cell death [10,33,34]. From these reports, the neuroprotective effects of NE-100 seem to be similar to these of BIX. Since p50 ATF6 is increased by NE-100, but not p90 ATF6, it is possible that NE-100 promotes the dissociation of GRP78 from ATF6, or activates the function of S1P and S2P. However, the mechanism by which NE-100 promotes cleavage of ATF6 is not understood, thus warranting further investigation. Moreover, NE-100 did not induce CHOP expression, as did tunicamycin (Fig. 2). In many previous reports, NE-100 is used as a selective sigma-1 receptor antagonist in order to antagonize the effects of sigma-1 receptor agonists [35–37]. Sigma-1 receptors show a protective effect against ER stress [38]. Moreover, we reported that a sigma-1 receptor agonist such as imipramine protects neuronal cells against ER stress-induced cell death [19]. It may raise the question that antagonists would have the opposite effect to agonists. If sigma-1 antagonists have the opposite effect of agonists, NE-100 and BD1047 should exacerbate the tunicamycin-induced cell death. However, NE-100, but not BD1047, protected cells, and the two antagonists never worsened tunicamycin-induced cell death. From the results, the sigma-1 antagonists may not have the opposite effect, namely the sigma-1 antagonists may not be inverse agonists. In addition, if the protective effect is involved in sigma-1 receptor antagonism, BD1047, another sigma-1 receptor antagonist, might show a protective effect against the tunicamycin-induced cell death as well as that of NE-100. However, BD1047 did not protect HT22 cells against the tunicamycin-induced cell death. Therefore, we speculated that the mechanism of protective effect of NE-100 was induction of GRP78 through ATF6 pathway- independent of sigma-1 receptor antagonism.

In conclusion, NE-100 suppressed the ER stress-induced cell death via CHOP expression, possibly by upregulation of GRP78, but not sigma-1 receptor.

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