**Abstract**

Oxidative stress, caused by the over production of reactive oxygen species (ROS), has been shown to contribute to cell damage associated with neurotrauma and neurodegenerative diseases. ROS mediates cell damage either through direct oxidation of lipids, proteins and DNA or by acting as signaling molecules to trigger cellular apoptotic pathways. The 78 kDa glucose-regulated protein (GRP78) is an ER chaperone that has been suggested to protect cells against ROS-induced damage. However, the protective mechanism of GRP78 remains unclear. In this study, we used C6 glioma cells transiently overexpressing GRP78 to investigate the protective effect of GRP78 against oxidative stress (hydrogen peroxide)-induced injury. Our results showed that the overexpression of GRP78 significantly protected cells from ROS-induced cell damage when compared to non-GRP78 overexpressing cells, which was most likely due to GRP78-overexpressing cells having higher levels of glutathione (GSH) and NAD(P)H:quinone oxidoreductase 1 (NQO1), two antioxidants that protect cells against oxidative stress. Although hydrogen peroxide treatment increased lipid peroxidation in non-GRP78 overexpressing cells, this increase was significantly reduced in GRP78-overexpressing cells. Overall, these results indicate that GRP78 plays an important role in protecting glial cells against oxidative stress via regulating the expression of GSH and NQO1.

**Introduction**

Reactive oxygen species (ROS) are one of the cytotoxic factors produced from damaged cells that cause oxidative stress and tissue damage during neurotrauma [1]. Hydrogen peroxide (H$_2$O$_2$), a ROS, is released from dying cells during neurotrauma and neurodegenerative disease and causes tissue destruction [2,3]. H$_2$O$_2$ can produce hydroxyl radicals (OH$^*$) and mediate cell damage either through direct oxidation of lipids, proteins and DNA or as a signaling molecule to trigger cellular apoptotic pathways [4–6]. Therefore, it is important to protect cells from H$_2$O$_2$-induced cell damage as a therapeutic strategy against neurotrauma and neurodegenerative diseases [7,8]. Endoplasmic reticulum (ER) stress has been reported to be one of the pathways via which cells are damaged and die following ROS exposure [9,10].

The mechanism by which ER stress promotes apoptosis in cells hinges on driving the accumulation of structurally abnormal proteins that are usually repaired by ER chaperones to prevent cell death [11]. The 78 kDa glucose-regulated protein (GRP78) is one example of an ER chaperone that regulates protein folding in the ER and controls the ER-Ca$^{2+}$ balance via trans membrane ER stress sensors, which contribute to cell survival [11–13]. GRP78 has been suggested to not only protect cells against high-concentrations of glutamate or tunicamycin, which induce ER stress directly [14], but also protect cells from ROS damage [15–17].

Many studies have focused on various antioxidant factors, such as glutathione and NAD(P)H:quinone oxidoreductase 1 (NQO1). A previous study reported that induction of NQO1 and GSH by dimethyl fumarate, 3H-1,2-dithiole-3-thione or tert-butylhydroquinone (tBHQ) protected against neurocytotoxicity caused by dopamine, 6-hydroxydopamine, 4-hydroxy-2-nonenal, or H$_2$O$_2$ [18]. As this study described, these antioxidants have recently been demonstrated to play an important role in protecting cells against oxidative stress [19–21]. Glutathione is the most abundant low molecular weight thiol in most organisms [20,22]. There are two types of glutathione, reduced glutathione (GSH) and oxidized glutathione (GSSG), depending on the environment. Reduced glutathione (GSH) is the main non-protein antioxidant and plays a critical role in the detoxification of H$_2$O$_2$ and lipid hydroperoxide, and is involved in the protection against oxidative stress [18]. Similarly, NQO1, one of the most extensively investigated phase 2 enzymes, is an effective antioxidant that protects membrane
phospholipids from oxidative damage and plays an important protective role in oxidative stress [19].

Few studies have investigated the influence of GRP78 on NQO1. Some studies have suggested that H₂O₂ may not be involved in ER stress-dependent cell damage because the response of GRP78 is different following H₂O₂ exposure and other cytotoxic factors [23,24]. Similarly, a report on PKE-like ER kinase (PERK), a ER-stress sensing protein that resides in the ER, suggested that the PERK pathway is activated after dissociation of GRP78 from PERK monomers and leads to intracellular GSH production [25]. As these studies showed, the role of GRP78 during oxidative stress remains unclear. Therefore, we used cells transiently overexpressing GRP78 to investigate the protective effect of GRP78 against high extracellular concentrations of H₂O₂ and evaluated the response of glutathione and NQO1.

**Materials and Methods**

**Cell Culture and Culture Conditions**

The rat C6 glioma cell line was obtained from American Type Culture Collections (ATCC, Manassas, VA, USA). Cells were plated onto 6 cm tissue culture dishes and maintained at 37°C with 5% (v/v) CO₂ and 95% (v/v) air. The culture medium consisted of Dulbecco’s modified Eagle’s medium (DMEM; Gibco/Life Technologies, Carlsbad, CA, USA), 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) penicillin–streptomycin.

**H₂O₂ Treatment of C6 Cells**

C6 cells were plated onto 6 cm tissue culture dishes at a density of 2.0 × 10⁵ cells/dish, incubated for 36 h and then treated with fresh medium containing 1–6 mM H₂O₂ (Wako, Osaka, Japan) for 6 and 24 h.

**Antibodies**

Antibodies used were: rabbit polyclonal antibody against GRP78 (AnaSpec Inc. Fremont, CA USA); goat polyclonal antibody against GRP78 (Santa Cruz Biotechnology, Inc. Dallas, TX, USA); rabbit polyclonal antibody against NAD(P)H:quinone oxidoreductase1 (NQO1) (Santa Cruz Biotechnology Inc.); mouse monoclonal antibody against NAD(P)H:quinone oxidoreductase1 (NQO1) (Novus Biologicals, LLC. Littleton, CO, USA); mouse monoclonal antibody against β-actin (Sigma-Aldrich Inc. St. Louis, MO, USA); Alexa 488-conjugated anti-rabbit antibody; Alexa 488-conjugated anti-mouse antibody (Invitrogen/Life Technologies); PE-conjugated anti-mouse secondary antibody

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**Figure 1.** Schematic illustration of the pIRES2-AcGFP1 plasmid constructs used in the present study. cDNA of the rat GRP78 carried the XhoI/PstI cut sites and was cloned into the pIRES2-AcGFP1 plasmid.

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**Figure 2.** GRP78 expression in C6 cells following treatment with H₂O₂. (A) FACS analysis revealed a significant increase in the number of AP-positive cells following treatment with H₂O₂ when compared with untreated C6 cells (*P < 0.05). Compared with untreated C6 cells, no significant differences were observed in GRP78 protein levels at any H₂O₂ concentration. (B) Western blot analysis of GRP78 in C6 cells treated with H₂O₂ for 6 h and 24 h. There were no obvious changes in GRP78 expression levels.

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Intracellular GRP78 and NQO1 Detection

The IntraStain reagent kit (DAKO) has previously been used successfully for the detection of intracellular GRP78 or NQO1 protein expression under conditions of tunicamycin treatment and β-actin [14]. Following exposure to treatment, cells were fixed and permeabilized using IntraStain according to the manufacturer’s instructions. For the detection of cytoplasmic GRP78 or NQO1, cells were stained with rabbit polyclonal anti-GRP78 or mouse monoclonal anti-NQO1, and then stained with the Alexa 488-conjugated anti-rabbit secondary antibody or PE-conjugated anti-mouse secondary antibody. GRP78 expression and β-actin expression were measured with a BD LSRFortessa™ apparatus (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using FlowJo™ Software (Tree Star Inc. Ashland, OR, USA).

Construction of Chimeric Proteins and Transfection

The pRES2-AcGFP1 plasmid was purchased from Clontech Laboratories. The plasmid contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus between the multiple cloning site (MCS) and the Aequorea coerulescens green florescent protein (Ac GFP) coding region. This permits both the gene of interest and the Ac GFP gene to be translated to a single bicistronic mRNA. A 1.9-kb cDNA of rat GRP78 (GenBank M14059), covering a whole open reading frame, was composed by Takara Bio, Inc. (Otsu, Japan) carrying Xhol/PstI cut sites and cloned into pIRES2-AcGFP1 plasmid (Fig. 1). The pIRES-AcGFP1 vector was designed so that cells transfected transiently express GFP and the protein. Therefore, cells transfected with the GRP78 gene and expressing the protein expressed GFP. C6 cells were transfected with the indicated plasmids using the Neon™ transfection system (Invitrogen/Life Technologies). After transfec-
tion, cells consisting of GFP-positive and -negative cells were plated onto 6 cm tissue culture dishes at a density of $2.0 \times 10^6$ cells/dish and incubated in DMEM with 10% (v/v) FBS for 36 h. Cells were then treated with fresh medium containing 1–6 mM of H$_2$O$_2$ for 6 h.

**Western Blot Analysis**

After 36 h of transfection, GFP-positive and -negative cells were sorted using the FACSAria™ apparatus (Becton Dickinson, San Jose, CA, USA). Total extracted proteins from cultured cells were quantified using the Lowry method. Equal amounts of total protein from each sample were loaded onto a 12.5% (w/v) SDS–polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were then incubated with the primary antibodies at 4°C overnight, followed by incubation with the HRP-linked anti-rabbit IgG (DAKO), anti-goat IgG (DAKO), or anti-mouse IgG (DAKO) antibodies for 1 h. Signals were detected using Immobilon Western Chemiluminescent HRP (Millipore). Intensity of specifically amplified products was quantified by densitometric scans of the films using computer software for a Macintosh “CS analyzer” (ATTO, Tokyo, Japan).

**Annexin V/Propidium Iodide (PI) Double Staining**

Cells undergoing apoptosis or necrosis can be stained and quantified using Annexin V and propidium iodide (PI). Cells treated with H$_2$O$_2$, were washed once with phosphate buffered saline (PBS), and stained for 15 min with allophycocyanin-conjugated (APC)–Annexin V and PI (Becton Dickinson), according to the manufacturer’s instructions. Quantification of apoptotic/necrotic cell-death staining with APC–annexin V and PI under each condition were measured with a BD LSRRfortessa™ apparatus (Becton Dickinson) and analyzed using FlowJo™ Software (Tree Star Inc. Ashland, OR, USA). Annexin V-negative/PI-negative cells were considered living cells.

**Evaluation of Lipid Peroxidation**

The lipid peroxidation of the cell membrane after H$_2$O$_2$ exposure was examined using cis-parinaric acid as a probe. Oxidation of this probe is accompanied by decreased fluorescence and absorption [26,27]. cis-Parinaric Acid® was purchased from Molecular Probes/Life Technologies and the method was performed with slight modification as described by Hedley et al. [26]. Briefly, at 5 h after incubation with H$_2$O$_2$, 10 μM of cis-
Parinaric Acid was added and the incubation was continued for 1 h (total incubation time was 6 h). Cells were collected with PBS and the fluorescence was measured using the BD LSRFortessa™ apparatus (Becton Dickinson) and analyzed using FlowJo™ Software (Tree Star Inc.) The excitation and emission wavelengths were 320 and 420 nm, respectively.

Figure 6. Evaluation of GRP78 overexpressing cells following H₂O exposure. FACS analysis of GFP-negative and -positive cells labeling positive for Annexin V/PI following treatment with H₂O₂. The percentage of GFP-positive cells that were AP-positive significantly decreased when compared with GFP-negative cells following treatment with 1, 3 and 6 mM H₂O₂ (*P<0.05).

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Parinaric Acid was added and the incubation was continued for 1 h (total incubation time was 6 h). Cells were collected with PBS and the fluorescence was measured using the BD LSRFortessa™ apparatus (Becton Dickinson) and analyzed using FlowJo™ Software (Tree Star Inc.) The excitation and emission wavelengths were 320 and 420 nm, respectively.

Figure 7. Evaluation of GRP78 overexpression on cell lipid peroxidation following H₂O₂ exposure. FACS analysis of the mean fluorescence intensity of cis-Parinaric acid in GFP-negative and -positive cells following treatment with H₂O₂. The cis-Parinaric Acid mean fluorescence intensity of GFP-positive cells was significantly higher when compared with GFP-negative cells following treatment with 1, 3 and 6 mM H₂O₂ (*P<0.05).

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GSH Assay

Cellular GSH levels were monitored and analyzed using the ThiolTracker Violet GSH detection reagent® (Molecular Probes/Life Technologies) according to the manufacturer’s protocol. Cells were seeded in 6 cm dishes, and treated with 1–6 mM H₂O₂ for 6 h. After designated treatment times, cells were washed with

Figure 8. GSH expression levels in GRP78 overexpressing cells following H₂O₂ treatment. FACS analysis of the mean fluorescence intensity of GSH in GFP-negative and -positive cells following treatment with H₂O₂. The GSH mean fluorescence intensity of GFP-positive cells was significantly higher when compared to GFP-negative cells following treatment with 1, 3 and 6 mM H₂O₂ (*P<0.05).

Figure 9. Evaluation of NQO1 expression levels in GRP78 overexpressing cells following H₂O₂ exposure. FACS analysis of the mean fluorescence intensity of NQO1 in GFP-negative and -positive cells following treatment with H₂O₂. The NQO1 mean fluorescence intensity of GFP-positive cells was significantly higher when compared to GFP-negative cells following treatment with 1, 3 and 6 mM H₂O₂ (*P<0.05).
GFP-negative cells and non-treated C6 cells (n = 3, P < 0.05), the fluorescence intensity of GSH significantly decreased following treatment at all concentrations of H2O2 when compared to untreated C6 cells (n = 5; P < 0.05) (Fig. 3A). This result indicated that H2O2 causes lipid peroxidation of the cell membrane. Similarly, the mean fluorescence intensity of GSH significantly decreased following treatment at all concentrations of H2O2 when compared with untreated C6 cells (n = 4; P < 0.05) (Fig. 3B). These results indicated that intracellular GSH expression levels reduced when cells were subjected to excessive oxidative stress.

Evaluation of cis-Parinaric Acid and GSH Following H2O2 Treatment

FACS analysis revealed that the mean fluorescence intensity of cis-Parinaric acid significantly decreased following treatment at all concentrations of H2O2 when compared to untreated C6 cells (n = 5; P < 0.05) (Fig. 3A). This result indicated that H2O2 causes lipid peroxidation of the cell membrane. Similarly, the mean fluorescence intensity of GSH significantly decreased following treatment at all concentrations of H2O2 when compared to untreated C6 cells (n = 4; P < 0.05) (Fig. 3B). These results indicated that intracellular GSH expression levels reduced when cells were subjected to excessive oxidative stress.

Expression of GRP78 Protein and NQO1 in GFP-positive Cells after Transfection

At 36 h post-transfection, an average of 43% of cells was GFP-positive (Fig. 4A). Quantitation of immunoblots of GFP-positive cell extracts revealed a significant increase in GRP78 protein levels when compared with GFP-negative cells and non-treated C6 cells (n = 5, P < 0.05) (Fig. 4B). This result showed that GFP-positive cells overexpress GRP78 protein before exposure to H2O2 when compared with GFP-negative cells and non-treated C6 cells. Moreover, we observed that NQO1 expression significantly increased in GFP-positive cells when compared with GFP-negative cells and non-treated C6 cells (n = 3, P < 0.05) (Fig. 5).

Evaluation of GRP78 Overexpressing Cells Following H2O2 Exposure

After transfection, a mixture of cells consisting of GFP-positive and -negative cells were incubated for 36 h with 10% (v/v) FBS/DMEM. Cells were then treated with 1–6 mM H2O2 for 6 h. To evaluate the protective effect of GRP78, apoptotic/necrotic cells were labeled with annexin V and PI, and the ratio of AP-positive cells (AP-positive cells) following 1–6 mM H2O2 treatment in the number of apoptotic/necrotic cells, namely annexin V/PI-negative cells (AP-positive cells) following 1–6 mM H2O2 treatment at 6–24 h compared to that in the controls (Fig. 2A). The number of GRP78-expressing cells following treatment with H2O2 did not significantly increase compared to that in untreated C6 cells at any concentration (Fig. 2A). Similarly, western blot analysis revealed that the expression of the GRP78 protein in the groups treated with 1–6 mM H2O2 for 6 and 24 h did not increase obviously when compared to untreated C6 cells (n = 3; Fig. 2B).

Results

Cell Damage and GRP78 Expression Following H2O2 Treatment

Flow cytometric (FACS) analysis revealed a significant increase in the number of apoptotic/necrotic cells, namely annexin V/PI-positive cells (AP-positive cells) following 1–6 mM H2O2 treatment at 6–24 h compared to that in the controls (Fig. 2A). The number of GRP78-expressing cells following treatment with H2O2 did not significantly increase compared to that in untreated C6 cells at any concentration (Fig. 2A). Similarly, western blot analysis revealed that the expression of the GRP78 protein in the groups treated with 1–6 mM H2O2 for 6 and 24 h did not increase obviously when compared to untreated C6 cells (n = 3; Fig. 2B).

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Discussion

GRP78 levels have been reported to increase in cells following cytotoxic-induced ER stress, where it contributes to cell survival [11,12,28]. According to reports that metals are implicated in the etiology or pathogenesis of Alzheimer’s disease, some metals such as lead (Pb) induce the expression of GRP78, which is often associated with oxidative stress, and Pb impairs GRP78 function following binding [29]. Moreover, it has been reported that GRP78 may play a role in the modulation of the sensitivity of cells to stress after oxidative injury. Increases in the mRNA expression of GRP78 are observed in retinal pigment epithelial cells exposed to oxidative stress [17]. Experiments using cultured neurons reveal that GRP78 may protect cells against oxidative stress via actions
involving mainly the maintenance of calcium homeostasis [16]. Meanwhile, it was reported that GRP78 expression did not increase when cells were exposed to H$_2$O$_2$, which suggested that H$_2$O$_2$ exposure may not induce the ER stress pathway [24]. In our study, an increase in GRP78 expression in C6 cells was not observed after treatment with H$_2$O$_2$, however, the viability of cells decreased. Considering previous reports, our results suggest that GRP78 itself could play an important role in protecting cells against H$_2$O$_2$ injury regardless of whether the pathways that mediate GRP78 expression respond to their extracellular stimuli.

H$_2$O$_2$ causes cytotoxicity via the formation of more potent oxidants including OH$^-$, which causes lipid peroxidation of the cell membrane [7,30]. Lipid peroxidation disrupts the normal structure of cellular and subcellular membranes. In addition, the process produces byproducts such as 4-hydroxynonenal (4-HNE) or acrolein, both of which bind to proteins and damage their structure and function [30,31]. The present results show that GRP78 overexpressing cells suppress lipid peroxidation and may contribute to cell survival following H$_2$O$_2$ treatment. These data suggest that GRP78 can promote the expression of some antioxidants and may contribute to the protection of cells against H$_2$O$_2$ injury.

While a number of antioxidants are involved in the detoxification of H$_2$O$_2$, GSH is the primary defense against H$_2$O$_2$ [18]. GSH inhibits lipid peroxidation initiation by scavenging OH$^-$ or other ROS. Moreover, GSH also serves as a co-factor for GSH peroxidases that remove H$_2$O$_2$ [20,22]. It was reported that GSH was useful for curtailment of lipid peroxidation damage in acute spinal cord injury [20]. The ratio of GSH reduces when an increase in ROS induces ER stress [31,32]. In our results, when cells were exposed to H$_2$O$_2$, GSH expression in GRP78 overexpressing cells increased in ROS induced by the formation of more potent oxidants.

Overexpression of GRP78 plays an important role in protecting glial cells against H$_2$O$_2$ toxicity by regulating GSH and NQO1 expression. However, there are several pathways and factors related to GRP78 expression in cells and further studies are required to understand the mechanisms involved and the direct relationship between GRP78, GSH and NQO1 in order for molecular/pharmacological treatments of neurotrauma or neurodegenerative diseases to be developed.

Author Contributions
Conceived and designed the experiments: K. Suyama K. Sakabe MW. Performed the experiments: K. Suyama YO. Analyzed the data: K. Suyama YO. Contributed reagents/materials/analysis tools: AO JM TT HT. Wrote the paper: K. Suyama.

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