Grp78 Suppresses Lipid Peroxidation Of Glial Cell Membrane By Oxidative Stress In Traumatic Spinal Cord Injury

Kaori Suyama, M.D., Ph.D.,1 Masahiko Watanabe, M.D., Ph.D.,2 Kou Sakabe, M.D., Ph.D.,1 Asako Otomo, Ph.D.,3 Yoshinori Okada, Ph.D.,4 Hayato Terayama, Ph.D.,1 Takeshi Imai, M.D.,2 Joji Mochida, M.D., Ph.D.2

1Dept. of Anatomy and Cellular Biology, Basic Medical Science, Tokai University School of Medicine, Isehara, Japan, 2Dept. of Orthopedic Surgery, Surgical Science, Tokai University School of Medicine, Isehara, Japan, 3Dept. of Molecular Life Science, Basic Medical Science, Tokai University School of Medicine, Isehara, Japan, 4Tokai University Teaching and Research Support Center, Isehara, Japan.

Disclosures:

Introduction: Reactive oxygen species (ROS) are among the cytotoxic factors produced from damaged cells that cause oxidative stress and tissue damage during neurotrauma including spinal cord injury (SCI). Hydrogen peroxide (H2O2), a ROS, is released from dying cells during SCI and causes tissue destruction. H2O2 can produce hydroxyl radicals (OH•) and mediate cell damage either through direct oxidation of lipids, proteins, and DNA or by acting as a signaling molecule to trigger cellular apoptotic pathways. Thus, it is important to protect cells from H2O2-induced cell damage as a therapeutic strategy against SCI. Endoplasmic reticulum (ER) stress has been reported to be one of the causes of cell damage and death following ROS exposure. 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. Several recent studies have focused on various antioxidant factors. Glutathione is the main non-protein antioxidant, plays a critical role in the detoxification of H2O2 and lipid hydroperoxide, and is involved in protection against oxidative stress. Therefore, we used cells transiently overexpressing GRP78 to investigate the protective effect of GRP78 against high extracellular concentrations of H2O2 and evaluated the glutathione response.

Methods: Cells of the rat C6 glioma cell line, were plated on 6 cm dishes at a density of 2.0×10^6 cells/dish, incubated for 24 h in DMEM with 10% FBS (v/v), and treated with H2O2(1, 3, and 6 mM) for 6 h. Apoptotic/necrotic cell-death was monitored using annexin V and propidium iodide (PI). Lipid peroxidation of the cell membrane was monitored using cis-parinaric acid. Oxidation of this probe is accompanied by decreased fluorescence and absorption. Cellular reduced glutathione (GSH) levels were monitored and analyzed using the ThiolTracker Violet GSH detection reagent® (Molecular Probes/Life Technologies). GRP78 expression was monitored by fixing and permeabilizing cells using the IntraStain® reagent kit. For the detection of cytoplasmic GRP78, cells were stained with anti-GRP78 antibody. Quantitation of Annexin V/PI positive cells, GRP78 expression, cis-parinaric acid fluorescence, and GSH expression under each condition was evaluated by flow cytometry (FACS). The cDNA of rat GRP78 (GenBank M14059), covering a whole open reading frame, was cloned into a pIRES2-AcGFP1 plasmid. The pIRES-AcGFP1 vector was designed to allow transfected cells to transiently express GFP and GRP78-protein. C6 cells were transfected with the indicated plasmid using the Neon™ transfection system. After transfection, a mixture of cells consisting of GFP-positive (GFP+) and -negative (GFP−) cells was plated onto culture dishes, incubated for 36 h, and treated with H2O2(1, 3, and 6 mM) for 6 h. At 36 h after transfection, GFP+ and GFP− were sorted and total extracted protein from each cell was quantified. Western blotting was used to determine the expression of GRP78 protein. The intensity of specifically amplified products was quantified by densitometric scans of films. As a positive control, cells were also probed with a mouse monoclonal antibody against β-actin. All data are expressed as the mean ± standard deviation (SD). Analysis of variance and Student’s t-test were used to assess the difference between the means of test samples and controls. Significance was set at P < 0.05.

Results: FACS analysis revealed a significant increase in the number of Annexin/PI-positive cells (AP+) following treatment at all concentrations of H2O2 compared with those of untreated cells. Compared with the control group, no significant differences were observed in the GRP78 protein levels at any H2O2 concentration. The mean fluorescence intensity of cis-parinaric acid significantly decreased following treatment at all concentrations of H2O2 compared with those of untreated cells, indicating that H2O2 causes lipid peroxidation of the cell membrane. At 36 h after transfection, an average of 41% of cells were GFP+. Quantitation of immunoblots revealed that GFP+ cells expressed GRP78 protein more highly than GFP− cells and non-treated C6 cells. The percentage of GFP+ cells that were AP+ significantly decreased compared with those of GFP− under all H2O2 conditions (Fig. 1) (n = 5, P < 0.05). The mean fluorescence intensity of cis-parinaric acid in GFP+ cells was significantly higher than that in GFP− cells under all H2O2 conditions (Fig. 2) (n = 5, P < 0.05). We observed GSH expression levels in glutathione by FACS. FACS revealed that the mean fluorescence intensity of GSH decreased in GFP− cells compared with GFP+ cells, following exposure to all concentrations of H2O2 (Fig. 3) (n = 5, P < 0.05).
**Discussion:** Our results revealed that the overexpression of GRP78 protected cells from H$_2$O$_2$-induced cell damage was significantly more than that of non-GRP78 overexpressing cells. Although H$_2$O$_2$ treatment increased lipid peroxidation in non-GRP78 overexpressing cells, this increase was significantly reduced in GRP78-overexpressing cells. GRP78 overexpressing cells were found to have higher levels of glutathione (GSH), an antioxidant that protects cells against oxidative stress. In conclusion, these results indicate that GRP78 plays an important role in protecting glial cells against oxidative stress, possibly by regulating the expression of GSH.

**Significance:** GRP78 is expected to protect cells against H$_2$O$_2$ injury by regulating antioxidants. Thus, GRP78 may be a potential therapeutic target for spinal cord injury.

**Acknowledgments:** This study was supported in part by the Research and Study Program of Tokai University Educational System General Research Organization.


**ORS 2014 Annual Meeting**
*Poster No: 1652*