Korean J Biol Sci 7: 265-269, 2003

Expression of Endoplasmic Reticulum Chaperone ERp29 in the Injured Spinal Cord

Soojung Park, Ho-Myung Hwang, Young-Ho Lee, Kwan-Hee You[1], Kee-Sun Shin[2] and O-Yu Kwon*

Department of Anatomy, College of Medicine, and [1]Department of Biology, College of Natural Sciences, Chungnam National University, Taejon 301-747, Korea;
[2]Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Korea

Key Words:
ERp29
Endoplasmic reticulum
Chaperone
Spinal cord Injury

Regulation of endoplasmic reticulum (ER) chaperone, ERp29, in traumatized rat spinal cord was investigated. Compared to the control, ERp29 expression was down-regulated at the lesion site 1 d after spinal cord injury. However, ERp29 expression gradually increased from 3 d after the injury and peaked remarkably after 7 d. Two ER chaperones (GRP94 and BiP) showed constantly strong expression levels 1 d after spinal cord injury while the expression levels of the other two (calnexin and PDI) were unchanged. In the case of ERp72, its expression level was increased 1 d after the injury and gradually decreased thereafter. This study suggests that ERp29 expression in the spinal cord after traumatic injury might be associated with the posttraumatic neural survival, playing a role as a molecular chaperone.

One of the most important functions of the endoplasmic reticulum (ER) is to provide the intracellular environment to facilitate the proper folding and assembly of newly synthesized secretory and membrane proteins in which numerous ER molecular chaperones reside and determine a protein's final destination (Gething and Sambrook, 1992; Ma and Hendershot, 2002). Recently, ERp29 cDNA encoding a 24.5 kDa soluble ER protein (p*I* 6.0) was almost simultaneously isolated from both the enamel epithelium and hepatoma cells (Demmer et al., 1997; Mkrtchian et al., 1998). Its gene expression was demonstrated in all mammalian cells studied, particularly in the secretory tissues such as the enamel epithelium, testes, lungs and spleen (Hubbard et al., 2000; Shnyder and Hubbard, 2002). The protein encoded by the ERp29 gene consists of two domains based on NMR structure data. The N-terminal domain of ERp29 resembles the thioredoxin module of the protein disulfide isomerase (PDI), although it does not contain the Cys-X-X-Cys motifs, which suggests a PDI-like redox function. The C-terminal domain represents a novel all-helical fold, which is absent in the human PDI (Liepinsh et al., 2001). The function of this domain remains unclear, even though its C-terminal has a KEEL sequence, which is a typical ER retention sequence for efficient retention of ER chaperones within that compartment (Munro and Pelham, 1987).

As with many ER chaperones that exhibit increased expression in response to ER stresses or disrupted protein synthesis in the ER, ERp29 can be induced by a variety of factors, i.e. the accumulation of malfolded proteins in the ER (Mkrtchian et al., 1987). Despite the vast amount of biochemical data on ERp29, its exact biological function is unclear. For example, ERp29 gene expression in the thyrocytes was transcriptionally regulated by thyroid-stimulating hormone (Kwon et al., 2000), and the ERp29 protein directly attends to thyroglobulin folding as an ER chaperone (Sargsyan et al., 2002). Enhanced expression of heat shock protein in response to spinal cord injury has already been reported (Gower et al., 1989; McIntosh and Raghupathi, 1995). This offers a possibility that this response might enhance posttraumatic neuronal survival. However, no direct data demonstrating that the ER chaperone is associated with spinal cord injury are available. In order to obtain a clue to ERp29 function, this study examined whether or not traumatic injury increases the ERp29 expression level such as and this is the first report showing that the expression of ER chaperones including ERp29 are induced after spinal cord injury.

Materials and Methods

Male Sprague-Dawley rats weighing 200-300 g were anesthetized using a mixture of halothane and a 1:2 flow ratio of NO_2/O_2. The backs of the animals were shaved

*To whom correspondence should be addressed.
Tel: 82-42-580-8206, Fax: 82-42-586-4800
E-mail: oykwon@cnu.ac.kr

and the skin was disinfected with a betadine solution. A midline longitudinal incision was made at the T12 and L2 level to expose the dorsal laminae and the spinous process under optical microscope. A laminectomy was performed at the L1 spinal cord level. Using a New York University (NYU) Impactor, a spinal cord injury was induced by dropping a 10 mg weight at a height of 20 mm. The muscle and skin were then sutured in layers. Subsequent to surgery, the lesioned rats were warmed and returned to their cages. Their bladders were emptied daily by manual pressure. In the control (sham-operated) rats, only a L1 laminectomy was performed.

The spinal cord was dissected and frozen at -80°C. The cord was then immediately homogenized in a lysis buffer (10 mM Tris-HCl, 2% SDS, 50 mM DTT, 2 mM EDTA, 0.02% bromphenol blue, and 6% glycerol, pH 6.8) and boiled for 1 min. After SDS-polyacrylamide gel electrophoresis, the proteins in the gel were transferred to an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad). The remaining binding sites on the membrane were blocked with 3% skim milk in T-PBS (10 mM sodium phosphate, 150 mM NaCl, and 0.05% Tween 20, pH 7.4) for 1 h. The membrane was then incubated with a 1:1000 dilution of the anti-chaperone antibodies (Gift from Dr. Paul Kim, University of Cincinnati Medical School) and a 1:500 dilution of the rabbit anti-ERp29 antibodies in T-PBS including 3% skim milk for 1 h. The polyclonal anti-ERp29 antibodies were generated by the synthetic peptides C-DTGYPYGEKQDEFKRLAENSASS-N' of the rat ERp29 (AnyGen Co, Kwangju, Korea). After washing several times with T-PBS, the membrane was incubated with either 0.1 μg/ml peroxidase-labeled goat anti-rabbit IgG or anti-mouse IgG in T-PBS for 1 h. The membrane was thoroughly washed with T-PBS and then with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4), followed by chemiluminescent detection using the ECL Western blotting Kit (Amersham).

The animals were anesthetized using pentothal sodium (50 mg/kg, i.p.) 1, 3 and 7 days after the traumatic spinal cord injury. Subsequently, they underwent transcardiac perfusion fixation with saline followed by 4% phosphate-buffered paraformaldehyde. The spinal cords were removed and postfixed in 4% paraformaldehyde overnight at 4°C. The spinal cord tissues were obtained, post-fixed in a cold 4% paraformaldehyde solution, and changed into a 30% sucrose solution at 4°C overnight. Frozen sections (30 μm in thickness, longitudinal section) of each tissue were prepared and collected in PBS in a 24-well plate. The sections were immersed for 30 min in 3% H_2O_2 in order to inactivate endogenous peroxidase. The sections were incubated for 1 h at room temperature in ERp29 antibodies (1:200) in 0.1 M PBS, pH 7.4, containing 0.1% Triton X-100, 1.5% bovine serum albumin (BSA), and 1:200 normal goat serum (NGS), followed by incubation for 1 h in 1:200 biotinylated goat anti-rabbit IgG (Vector) and 1:200 NGS in PBS. The immunoreactions were visualized by incubation for 1 h at room temperature in avidin-biotin-peroxidase complex (1:100, ABC Kit, Vector) in PBS, and for 5-10 min in 0.05% 3,3'-diaminobenzidine (DAB) and 0.01% H_2O_2 in 0.1 M PBS. Each sample section was mounted on a gelatin-coated slide, dehydrated, and coverslipped with a permount for viewing.

Results

As shown from the result of Western blot analysis (Fig. 1), regulation of the ER chaperones including ERp29

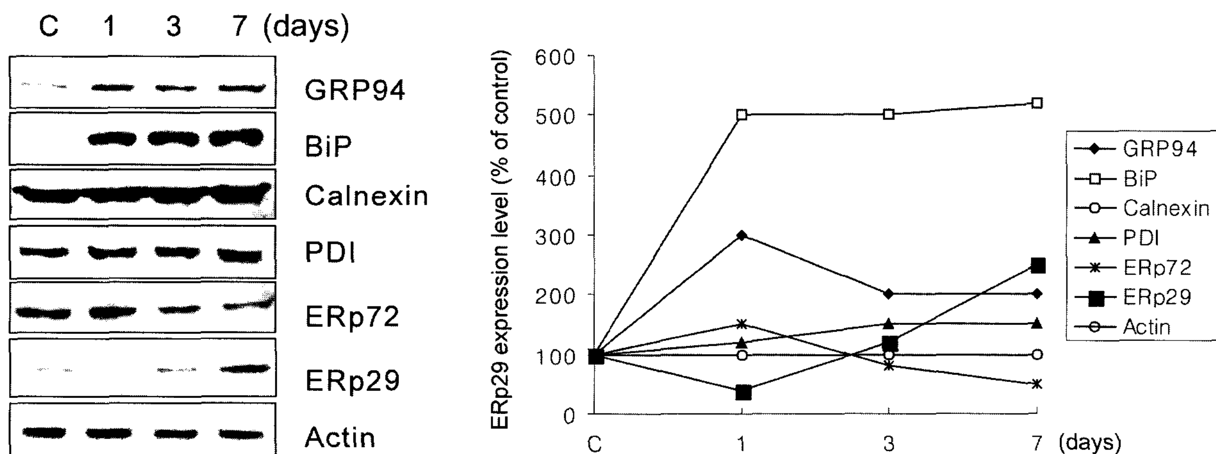

Fig. 1. Expression of ER chaperones by spinal cord injury. The spinal cord was dissected and frozen at -80°C and immediately homogenized in lyses buffer. The total lysate was prepared and analyzed by Western blot with a 1:1000 dilution of the anti-chaperone antibodies (GRP94, BiP, Calnexin, PDI, and ERp72) and a 1:500 dilution of the rabbit anti-ERp29 antibodies with a chemiluminescent detection using an ECL Western blotting Kit (Amersham). The mouse anti-actin antibodies were used as control. The experiments were performed with duplicate samples and the results were average of repeated experiments. The control value of each chaperone expression was calculated as 100%.

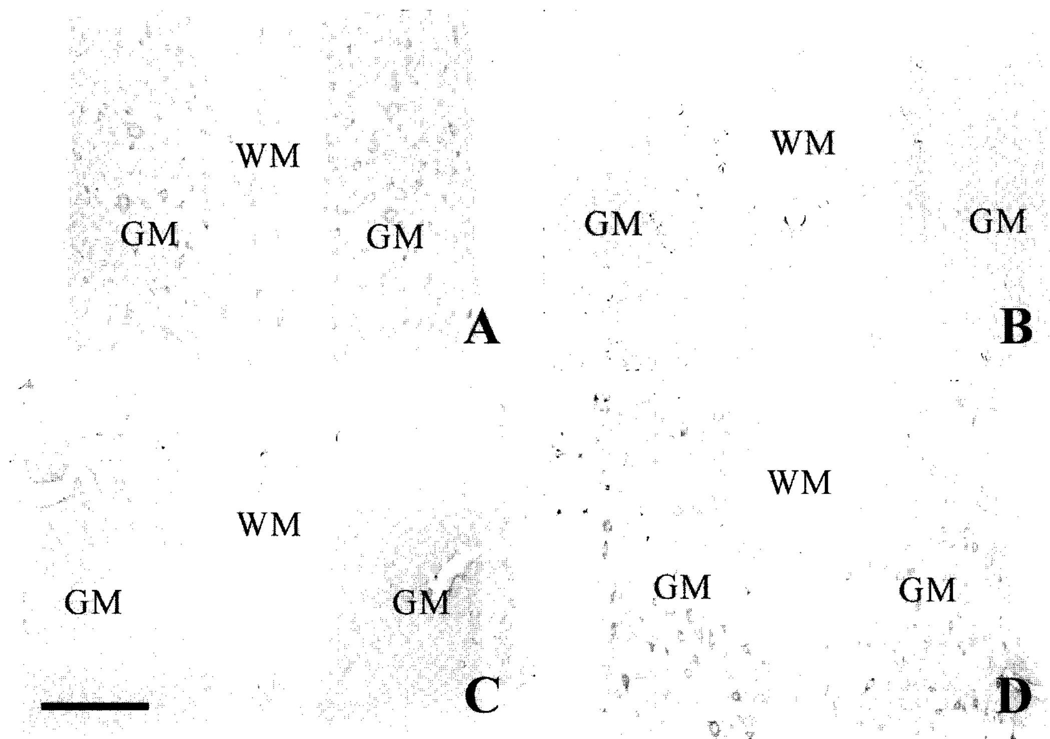

Fig. 2. ERp29 immunoreactivities in the spinal cords after traumatic spinal cord injury. Moderate ERp29 immunoreactive cells present in the gray matter (GM), and not in white matter (WM) in the control rats (A). Distinctive ERp29 immunoreactive cells were not observed in the spinal cord 1 d. (B) and 3 d (C). After the spinal cord injury. Strong ERp29 immunoreactive cells present in the gray matter (GM) 7 d after the spinal cord injury (D). Scale bar=100 μm.

was tested in injured spinal cord. Both ER chaperones, GRP94 and BiP, were immediately up-regulated 1 d after spinal cord injury, and this expression level was maintained for 7 d. In contrast, no obvious differences in the expression levels were found in the ER chaperones Calnexin and PDI 7 d after spinal cord injury. The ERp72 expression level was higher after 1 d spinal cord injury, which gradually decreased thereafter. On the other hand, ERp29 was down-regulated 1 d after the spinal cord injury compared to its control, after which its expression gradually increased for 7 d.

To confirm the result of Western blotting shown above, immunohistochemistry was performed against same samples using ERp29 antibody. The ERp29 immunoreactivity was moderately observed in the cells of the gray matter in control rats (Fig. 2A). The number of ERp29 immunoreactive cells had decreased significantly in the gray matter 1 and 3 d after the injury (Fig. 2B and 2C). However, ERp29 immunoreactivity was detected strongly in the gray matter neurons 7 d after the injury (Fig. 2D). ERp29 immunoreactive cells were not observed in the white matter of the control or experimental rats (Fig. 2A-D).

Discussion

Previous studies have identified several spinal cord injury-induced genes and proteins using several methods such as RT-PCR, Western blotting and immunohistochemistry. Gower et al (1989) first demonstrated that several types of heat shock proteins were induced following spinal cord injury in rats. Recently, Song et al. (2001) reported that many genes are associated with spinal cord injury using a DNA chip in which two heat shock proteins, HSP70 and heme oxygenase-1, were found. However, there is a paucity of data showing involvement of the ER chaperones in mechanical stress, particularly traumatic spinal cord injury.

ERp29, an ER chaperone, is mainly expressed in the secretory tissues and is sensitive to ER stresses (Shnyder and Hubbard, 2002). In addition, when rats were treated with a hepatotoxic dose of bromobenzene, ERp29 expression was remarkably up-regulated (Koen and Hanzlik, 2002). It has been suggested that ERp29 functions as a chaperone for protein maturation and/or stress-defense in the ER. The Hubbard group demonstrated that the ERp29 expression pattern was quite similar to PDI. However, the ratio of ERp29/PDI

was strikingly different in various cell lines tested, which suggests that ERp29 has functional roles independent of PDI (Hubbard et al., 2000).

In general, expression of the ER chaperones showed a very early response to ER stresses (Melnick et al., 1994). In this study, the ER chaperones GRP94 and BiP were strongly expressed 1 d after the spinal cord injury. In contrast, no obvious difference in the expression levels was found in calnexin (a membrane-bound chaperone) and PDI (a luminal ER foldase) until 7 d after the spinal cord injury. It is not surprising that ER chaperones (GRP94 and BiP) were dramatically increased in expression against spinal cord injury, since ER chaperones should recognize unfolded proteins that are over-produced by spinal cord injury. However, the unchanged expression levels of calnexin and PDI suggests that ER stress of spinal cord injury does not induce dramatic increase in ER size, because ER expansion is a known feature of unfolded protein accumulation in the ER. In the case of ERp72, although its expression level increased 1 d after the spinal cord injury, it gradually decreased thereafter. Compared to the other ER chaperones, different expression pattern was shown in ERp29: the down-regulation 1 d after the spinal cord trauma. Its expression level increased gradually 3 d after the spinal cord injury and peaked at 7 d.

Traumatic injury to the spinal cord triggers several secondary effects, including oxidative stress and compromised energy metabolism, which plays an important role in the biochemical and pathological changes in the spinal cord (Juurlink and Paterson, 1998). Recently published reports associated with the ER suggest that a reduced ERp29 level accompanies LPS-induced apoptosis and that ERp29 is a ubiquitous resident of the ER with a distinct role in secretory protein production (Huang et al., 2002). The ERp29 immunoreactive cells were lower in the gray matter 1 and 3 d after the traumatic spinal cord injury. This suggests that ERp29 may be down-regulated by oxidative stress in the early stage of traumatic spinal cord injury. The spinal cord has a great potential for recovery after injury. In these experiments, the functional recovery of locomotion was prominent in the hindlimb (data not shown), and strong ERp29-immunoreactive cells appeared in the neurons in the gray matter 7 d after the spinal cord injury.

The results of this study raise the possibility that ERp29 expression is a response to a spinal cord injury for posttraumatic neural survival and/or repair. Therefore, an ERp29 study may provide a useful opportunity for the pathophysiology of a spinal cord injury. Recently, the *Drosophila* ERp29 homologue, *windbeute1,* which determines the ventral side of the embryo, was characterized suggesting that ERp29 may have an important biological function beyond mammals (Konsolaki and Schupbach, 1998). Overall, the results suggest that ERp29 may be involved in the functional recovery via secretory protein production after a traumatic spinal cord injury.

Acknowledgments

This study was supported by a grant from BioGreen21 program, Rural Development Administration of Korea.

References

Demmer J, Zhou C, and Hubbard MJ (1997) Molecular cloning of ERp29, a novel and widely expressed resident of the endoplasmic reticulum. *FEBS Lett* 402: 145-150.

Gething MJ and Sambrook J (1992) Protein folding in the cell. *Nature* 355: 33-45.

Gower DJ, Hollman C, Lee KS, and Tytell M (1989) Spinal cord injury and the stress protein response. *J Neurosurg* 70: 605-611.

Huang YH, Chang AY, Huang CM, Huang SW, and Chan SH (2002) Proteomic analysis of lipopolysaccharide-induced apoptosis in PC12 cells. *Proteomics* 2: 1220-1228.

Hubbard MJ, McHugh NJ, and Carne DL (2000) Isolation of ERp29, a novel endoplasmic reticulum protein, from rat enamel cells: evidence for a unique role in secretory-protein synthesis. *Eur J Biochem* 267: 1945-1957.

Juurlink BH and Paterson PG (1998) Review of oxidative stress in brain and spinal cord injury: suggestions for pharmacological and nutritional management strategies. *J Spinal Cord Med* 21: 309-334.

Koen YM and Hanzlik RP (2002) Identification of seven proteins in the endoplasmic reticulum as targets for reactive metabolites of bromobenzene. *Chem Res Toxicol* 15: 699-706.

Konsolaki M and Schupbach T (1998) *windbeutel*, a gene required for dorsoventral patterning in *Drosophila*, encodes a protein that has homologies to vertebrate proteins of the endoplasmic reticulum. *Genes Dev* 12: 120-131.

Kwon OY, Park S, Lee W, You KH, Kim H, and Shong M (2000) TSH regulates a gene expression encoding ERp29, an endoplasmic reticulum stress protein, in the thyrocytes of FRTL-5 cells. *FEBS Lett* 475: 27-30.

Liepinsh E, Baryshev M, Sharipo A, Ingelman-Sundberg M, Otting G, and Mkrtchian S (2001) Thioredoxin fold as homodimerization module in the putative chaperone ERp29: NMR structures of the domains and experimental model of the 51 kDa dimmer. *Structure* 9: 457-471.

Ma Y and Hendershot LM (2002) The mammalian endoplasmic reticulum as a sensor for cellular stress. *Cell Stress Chaperones* 7: 222-229.

McIntosh TK and Raghupathi R (1995) Cell stress genes and acute CNS injury. *Neuropathol Appl Neurobiol* 21: 477-479.

Melnick J, Dul JL, and Argon Y (1994) Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. *Nature* 370: 373-375.

Mkrtchian S, Fang C, Hellman U, and Ingelman-Sundberg M (1998) A stress-inducible rat liver endoplasmic reticulum protein, ERp29. *Eur J Biochem* 251: 304-313.

Munro S and Pelham HR (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48: 899-907.

Sargsyan E, Baryshev M, Szekely L, Sharipo A, and Mkrtchian S (2002) Identification of ERp29, an endoplasmic reticulum

lumenal protein, as a new member of the thyroglobulin folding complex. *J Biol Chem* 277: 17009-17015.

Shnyder SD and Hubbard MJ (2002) ERp29 is a ubiquitous resident of the endoplasmic reticulum with a distinct role in secretory protein production. *J Histochem Cytochem* 50: 557-566.

Song G, Cechvala C, Resnick DK, Dempsey RJ, and Rao VL (2001) Gene chip analysis after acute spinal cord injury in rat. *J Neurochem* 79: 804-815.

[Received June 18, 2003; accepted August 14, 2003]