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ENDOPLASMIC RETICULUM (ER) STRESS PROTEIN RESPONSES IN RELATION TO SPATIO-TEMPORAL DYNAMICS OF ASTROGLIAL RESPONSES TO STATUS EPILEPTICUS IN RATS

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Abstract—In the present study, we investigated whether endoplasmic reticulum (ER) stress is associated with neuronal- and astroglial-death in the hippocampus using LiCl-pilocarpine-induced status epilepticus (SE) rat model. Glucose-related protein (GRP) 78 and protein disulfide isomerase (PDI) expressions were transiently increased in CA1 neurons and dentate granule cells, and subsequently decreased in these cells following SE. GRP94 and calnexin (CNX) expression was gradually reduced in CA1 neurons, not in dentate granule cells. Phospho-protein kinase RNA (PKR)-like ER kinase (pPERK), phospho-eukaryotic initiation factor 2α (peIF2A) and CCAAT/enhancer-binding protein homologous protein (CHOP) immunoreactivities were observed in 17%, 12% and 7% of degenerating CA1 neurons, respectively. GRP 78 and PDI expressions were also up-regulated in reactive astrocytes within the CA1–3 regions. In the molecular layer of the dentate gyrus, PDI-positive astrocytes showed TUNEL signal, nuclear apoptosis inducing factor translocation and pPERK/peIF2A/CHOP immunoreactivities. Four weeks after SE, clasmatodendritic astrocytes showed pPERK peIF2A and CNX immunoreactivities without CHOP expression. These findings indicate that SE-induced ER stress may be associated with astroglial apoptosis and autophagic astroglial death in the regional-specific pattern.

Key words: GRP78, GRP94, calnexin, protein disulfide isomerase, status epilepticus.

INTRODUCTION

Endoplasmic reticulum (ER) is a cell organelle that regulates glycosylation, folding and assembly of newly synthesized proteins. Stressful conditions such as oxidative stress, hypoxia and changed glucose metabolism disturb ER function due to the accumulation of unfolded proteins or changes in calcium homeostasis, which is collectively termed ER stress (Verkhratsky, 2005). ER stress induces the expressions of chaperones, attenuation of protein translation and activation of ER-associated degradation through the activation of ER sensor proteins including protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring protein 1 and activating transcription factor 6 (Bertolotti et al., 2000; Boysen et al., 2006). Four phases in response to ER stress have been identified (Yoshida et al., 2003). The first phase attenuates protein translation to reduce newly synthesized proteins. In the second phase, ER chaperones such as glucose-related protein (GRP) 78 increase protein-folding activity. In the third phase, unfolded or misfolded proteins are degraded by the ubiquitin–proteasome system in the cytosol. In the last phase, cell death pathways are activated when ER stress is too severe to repair ER function. Interestingly, prolonged ER stress elicits two different cell death pathways: apoptosis and autophagy. PERK activation phosphorylates eukaryotic initiation factor 2α (eIF2A) to attenuate new protein translation as a pro-survival mechanism. However, this transient event promotes the expression of CCAAT/enhancer-binding protein homologous protein (CHOP) and induces mitochondrial apoptosis (Rzymski and Harris, 2007). On the other hand, ER stress also activates autophagy to relieve cell stress as the compensatory pathway for ER-associated degradation via PERK signaling pathway (Yorimitsu and Klionsky, 2007; Rzymski et al., 2010). However, excessive autophagy triggers neuronal death (Koike et al., 2008). Therefore, ER stress enables the induction of distinct cell death pathways in response to various harmful stimuli.

Status epilepticus (SE), a prolonged seizure activity, is one of the emergency conditions with a high morbidity and mortality, and of epileptogenic episodes (Kim et al., 2011). Furthermore, a number of studies have demonstrated that SE results in regional-specific astroglial damage/death as well as neuronal death (Kang et al., 2006; Kim et al., 2008, 2011, 2014). Briefly, TUNEL-positive astroglial death is observed in the molecular layer (not

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Abbreviations: AIF, apoptosis-inducing factor; CHOP, CCAAT/enhancer-binding protein homologous protein; CNX, calnexin; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; eIF2A, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; GRP, glucose-related protein; PB, phosphate buffer; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; peIF2A, phospho-eukaryotic initiation factor 2α; PKR, protein kinase RNA; pPERK, phospho-protein kinase RNA (PKR)-like ER kinase; SE, status epilepticus.

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the hilus) of the dentate gyrus within the first week after pilocarpine-induced SE (SE, Kang et al., 2006; Kim et al., 2008, 2010). In contrast, vacuolized astroglial degeneration is detected within the stratum radiatum of the CA1 region in the late state period after SE (Kim et al., 2008, 2009, 2011). This fundamental change of injured astrocytes is first report by Alzheimer in 1910, which includes extensive swelling (hypertrophy) and vacuolization of cell bodies with disintegrated and beaded processes, and termed ''clasmatodendrosis'' by Cajal (Penfield, 1928). Sugawara et al. (2002) reported that astroglial vacuolization would be irreversible necrotic changes with TUNEL negativity. Recently, we have reported that clasmatodendrosis is a lysosome-derived autophagic astroglial death in response to epileptic seizures (Ryu et al., 2011a,b). Although these differential responses and susceptibilities to dysfunction and degeneration of astrocytes are most likely leading to aberrant neuronal functions or altered microenvironments, less defined are the mechanisms of astroglial death.

With respect to the characteristics of ER stress during cell survival or death, implications of ER stress in the cell death induced by SE would be useful to understand the molecular events in the pathophysiology of epileptogenic process. However, the role of ER stress upon cell death induced by SE is still controversial. Some investigators reported that ER stress involves neuronal death (Yamamoto et al., 2006; Sokka et al., 2007; Chen et al., 2013, 2014), or plays as a pro-survival mechanism in response to seizures (Kitao et al., 2001; Torres-Peraza et al., 2013). Therefore, this study was designed to elucidate the profile of various ER stress-related molecules and their relationships to neuronal death and astroglial damage in the rat hippocampus following pilocarpine-induced SE.

EXPERIMENTAL PROCEDURES

Experimental animals and chemicals

This study utilized the progeny of male Sprague–Dawley (SD) rats (7 weeks old) obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea. Animals were provided with a commercial diet and water *ad libitum* under controlled temperature, humidity and lighting conditions (22 ± 2 °C, 55 ± 5% and a 12:12 light/dark cycle with lights). Procedures involving animals and their care were conducted in accord with our institutional guidelines that comply with NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996). In addition, all possible efforts were taken to avoid animals' suffering and to minimize the number of animals used at each stage of the experiment. All reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA), except as noted.

SE induction

Animals were given LiCl (3 mEq/kg, i.p.) 24 h prior to the pilocarpine treatment. Animals were intraperitoneally (i.p.) treated with pilocarpine (30 mg/kg) 20 min after atropine methylbromide (5 mg/kg, i.p.). Within 20 to 30 min of the administration of pilocarpine, animals showed seizure activity between 2 and 5 on the Racine scale (including akinesia, facial automatisms, and limbic seizures consisting of forelimb clonus with rearing, salivation, masticatory jaw movements and falling). SE was defined as continuous grade 5 seizures (rearing, falling and loss of postural control) for more than 30 min without regaining normal behavior between the seizures. The assessment of the SE severity was based on the latency of the first behavioral seizure, the development of stage 5 seizures and the seizure score. Only animals showing SE were used in the present study. Diazepam (Valium, Hoffman Ia Roche, Neuilly sur-Seine, France; 10 mg/kg, i.p.) was administered 2 h after onset of SE and repeated, as needed. Control animals received saline in place of pilocarpine.

Tissue processing

Under urethane anesthesia (1.5 g/kg, i.p.), animals were perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at the designated time points (Non-SE, 12 h, 1 day, 3 days, 4 days, 1 week and 4 weeks after SE, $n = 5$, respectively). The brains were removed, postfixed for 24 h and rinsed in PB containing 30% sucrose at 4 °C for 2 days. Thereafter, the brains were frozen and sectioned with a cryostat at 30 μm and consecutive sections were collected in six-well plates containing PBS. For western blot, animals were decapitated under urethane anesthesia (1.5 g/kg, i.p.). The hippocampus was removed and homogenized in 50 mM Tris containing 50 mM HEPES (pH 7.4), EGTA (pH 8.0), 0.2% Tergitol type NP-40, 10 mM EDTA (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). After centrifugation, the protein concentration in the supernatant was determined using a Micro BCA Protein Assay Kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, USA).

Double immunofluorescence

Table 1 is a list of the primary antibodies used in the present study. Sections were incubated with 3% bovine serum albumin in PBS for 30 min at room temperature. Sections were then incubated in a mixture of each primary antibody in PBS containing 0.3% Triton X-100 and 2% normal chicken serum overnight at room temperature. After washing three times for 10 min with PBS, sections were also incubated in a mixture of FITC- and Cy3-conjugated secondary antisera (Amersham, Piscataway, NJ, USA, 1:200) for 1 h at room temperature. Sections were mounted in Vectashield mounting media with/without DAPI (Vector). For negative control, the hippocampal tissues obtained from non-SE and post-SE animals were incubated with pre-immune serum instead of primary antibody (AIF, CHOP,