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CONVERGENCE OF STRESS GRANULES AND PROTEIN AGGREGATES IN HIPPOCAMPAL CORNU AMMONIS 1 AT LATER REPERFUSION FOLLOWING GLOBAL BRAIN ISCHEMIA

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Abstract—The delayed and selective vulnerability of post-ischemic hippocampal cornu ammonis (CA) 1 pyramidal neurons correlates with a lack of recovery of normal protein synthesis. Recent evidence implicates sequestration of translational machinery into protein aggregates and stress granules as factors underlying persistent translation arrest in CA1 neurons. However, the relationship between protein aggregates and stress granules during brain reperfusion is unknown. Here we investigated the colocalization of protein aggregates and stress granules using immunofluorescence microscopy and pair-wise double labeling for ubiquitin/T cell internal antigen (TIA-1), ubiquitin/small ribosomal subunit protein 6 (S6), and TIA-1/S6. We evaluated the rat dorsal hippocampus at 1, 2 or 3 days of reperfusion following a 10 min global brain ischemic insult. At 1 day of reperfusion, ubiquitin-containing aggregates (ubi-protein clusters) occurred in neurons but did not colocalize with stress granules. At 2 days' reperfusion, only in CA1, cytoplasmic protein aggregates colocalized with stress granules, and ubiquitin-containing inclusions accumulated in the nuclei of CA1 pyramidal neurons. Functionally, a convergence of stress granules and protein aggregates would be expected to sustain translation arrest and inhibit clearance of ubiquitinated proteins, both factors expected to contribute to CA1 pyramidal neuron vulnerability.

Key words: brain ischemia and reperfusion, protein aggregates, stress granules, TIA-1, translation arrest, ubiquitin.

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Abbreviations: CA, cornu ammonis (Ammon's horn); CF, cytosolic fraction; eIF2α, alpha subunit of eukaryotic initiation factor 2; EM, electron microscopy; HSP70, 70 kDa heat shock protein; IF, insoluble fraction; I/R, ischemia and reperfusion; MAP, mean arterial pressure; NF, nucleoplasmic fraction; NIC, nonischemic control; PA, protein aggregate; SG, stress granule; S6, small ribosomal subunit protein 6; TIA-1, T cell internal antigen; ubi-protein cluster, ubiquitin-containing clusters of misfolded/damaged protein; 1DR, 1 day of reperfusion, 10 min ischemia plus 24 h reperfusion; 2DR, 2 days of reperfusion, 10 min ischemia plus 48 h reperfusion; 2VO/HT, bilateral carotid artery (two-vessel) occlusion and hypovolemic hypotension; 3DR, 3 days of reperfusion, 10 min ischemia plus 72 h reperfusion; 40S, small ribosomal subunit.

A lack of recovery of normal protein synthesis correlates with the selective death of hippocampal cornu ammonis (CA) 1 neurons following global brain ischemia and reperfusion (I/R) (Hossmann, 1993). Recent evidence points to the partitioning of ribosomes into translationally nonfunctional subcellular structures, including protein aggregates (PAs) and stress granules (SGs), as potential causes for the lack of recovery of normal protein synthesis in reperfused CA1 (reviewed in DeGracia and Hu, 2006).

PAs form as a result of I/R damage to intracellular proteins. Initially in reperfusion, ubiquitin-containing clusters of misfolded/damaged proteins (ubi-protein clusters) occur in all post-ischemic neurons. Ubi-protein clusters transform, by as yet undetermined mechanisms, into PAs, the latter of which persist exclusively in CA1 until the point of CA1 pyramidal cell death (Hu et al., 2000, 2001; Liu et al., 2005a,b). The process of cotranslational aggregation links PAs to CA1 translation arrest. Here, dysfunction of cotranslational chaperone activity following I/R is postulated to result in misfolding of nascent peptides, leading to their aggregation and to the subsequent trapping of ribosomes and other translation factors in insoluble complexes (Liu et al., 2005b).

SGs are punctate cytoplasmic structures that form in response to a variety of cellular stresses following the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 [eIF2α; phospho-form, eIF2α(P)] (Kedersha et al., 1999; reviewed in Kedersha and Anderson, 2002). SGs contain the small ribosomal subunit (40S), several initiation factors, poly-adenylated mRNAs, and several mRNA binding proteins including T cell internal antigen (TIA-1) and HuR (reviewed in Anderson and Kedersha, 2006). SGs are thought to sequester and protect mRNAs during periods of stress-induced translation arrest (Nover et al., 1989), and to perform functions related to cytoplasmic mRNA metabolism (Anderson and Kedersha, 2006). Following global I/R, small ribosomal subunit protein 6 (S6), as a marker of the 40S, was observed to be exclusively sequestered in SGs only in CA1 at 4 h reperfusion following resuscitation from a 10 min cardiac arrest (Kayali et al., 2005).

Hence, both PAs and SGs can plausibly contribute to the prolongation of translation arrest in CA1 pyramidal neurons. However, the question remains if there is a relationship between SGs and PAs. Each may contribute to translation arrest in parallel, but an interaction between PAs and SGs has not been ruled out (DeGracia and Hu, 2006). Thus, we report here studies of pair-wise colocalization of ubiquitin, a marker of PAs, and TIA-1 and the 40S protein S6, as markers of SGs. Our main finding was

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that, at 2 days of reperfusion, exclusively in CA1 pyramidal neurons, PAs colocalized with SGs. Functionally, a convergence of SGs and PAs would be expected to sustain translation arrest and prevent clearance of PAs, and both of these factors may contribute to the selective vulnerability of CA1 pyramidal neurons.

EXPERIMENTAL PROCEDURES

Materials

TIA-1 goat IgG primary antiserum (sc-1751) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse monoclonal S6 ribosomal protein antiserum (#2317) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit polyclonal antibody to ubiquitin (RPCA-Ubi) was purchased from Proteome Resources, L.L.C. (Englewood, CO, USA). Alexa FluorR 488 donkey anti-goat IgG, Alexa FluorR 555 donkey anti-rabbit IgG and Alexa FluorR 555 anti-mouse IgG were purchased from Molecular Probes (Eugene, OR, USA). Fluoro-Jade was purchased from Histochem, Inc. (Jefferson, AR, USA). All other chemicals were reagent grade.

Animal groups

All animal experiments were approved by the Wayne State University Animal Investigation Committee and were conducted following the *Guide for the Care and Use of Laboratory Animals* (National Research Council, revised 1996). All efforts were made to reduce animal suffering and minimize the total number of animals used. Global forebrain ischemia was induced using the bilateral carotid artery (two-vessel) occlusion and hypovolemic hypotension (2VO/HT) model of Smith et al. (1984). Male Long Evans rats (275–300 g) were initially anesthetized with 5% halothane, and anesthesia was maintained at 2% halothane in 100% O_2 using a facemask through the duration of the experiment. Rectal temperature was maintained at 37±0.5 °C by a homeostatic blanket system (Harvard Apparatus, Holliston, MA, USA) during ischemia and for the first 1 h of reperfusion. Mean arterial pressure (MAP) was monitored in real time via tail artery access. The common carotids were isolated and lassoed bilaterally. Blood gas measurements ensured pH=7.4±0.1, pO_2>80 mm Hg and pCO_2=35±5 mm Hg immediately prior to the initiation of ischemia. Blood was withdrawn, via femoral arterial access, into a 10 ml syringe to MAP=50 mm Hg and carotids were clamped using microaneurysm clips. Blood was further withdrawn to maintain MAP at 40 mm Hg for the 10 min duration of ischemia. Following ischemia, blood was reinfused at a rate of 5 ml/min. All cut-down wounds were sutured, and anesthesia and temperature control were maintained for 1 h following surgery. Post-surgical animals were housed in a 12-h light/dark cycle and provided food and water access during the reperfusion period. Animals displaying frank necrosis, weight loss >15% initial body weight/day, or sustained seizure activity were excluded from the study. Our overall survival rate for the reperfused groups was 75%. Experimental groups (n=3 per group) were: nonischemic controls (NIC), 10 min ischemia and 24 h reperfusion (1 day of reperfusion, 1DR), 10 min ischemia and 48 h reperfusion (2 days of reperfusion, 2DR), and 10 min ischemia and 72 h reperfusion (3 days of reperfusion, 3DR).

At appropriate times, animals were transcardially perfused, and 50 μm slices through the dorsal hippocampus were obtained via vibratome and stored at −20 °C in cryostat solution until used, as previously described (DeGracia et al., 2006).

General cell staining

For Toluidine Blue staining, slide-mounted slices were washed ×3 in 1× PBS and air dried overnight. Sections were dehydrated in a graded ethanol series and then incubated in a 10% Toluidine Blue in 100% ethanol solution for 1 h at room temperature. Slides were washed in a graded ethanol series followed by 100% xylene. Slides were then coverslipped with Permount for viewing. Fluoro-Jade staining was performed exactly as described in Schmued et al. (1997). Fluoro-Jade slides were examined using FITC excitation and emission wavelengths.

Immunohistochemistry

Double-labeling immunohistochemistry was performed as previously described (Kayali et al., 2005; DeGracia et al., 2006). Pairwise double staining of ubiquitin and TIA-1, ubiquitin and S6, and TIA-1 and S6 was performed on adjacent, 50 μm tissue sections. Primary antisera dilutions were: anti-TIA-1, 1:100; anti-S6, 1:50; anti-ubiquitin, 1:200. Secondary antisera dilutions were 1:300 in 1% normal donkey serum in PBS containing 0.3% Triton X-100. Slides were examined on an Axioplan 2 Imaging System (Carl Zeiss, Oberkochen, Germany) equipped with an ApoTome, and optical sectioning was performed using the ×63 oil immersion objective to generate z-stacks as previously described (Kayali et al., 2005; DeGracia et al., 2006). Fluorescent micrographs shown in the figures are orthographic projections of 3.75 μm z-stacks (15×0.25 μm optical sections) unless otherwise stated.

Quantitative analysis of colocalization

"Yellow channels" were constructed from orthographic projections of acquired z-stacks, and colocalization, in discrete spots, of paired antigens was quantified in BioImage Intelligent Quantifier (BioImage, Jackson, MI, USA), as previously described (Kayali et al., 2005; DeGracia et al., 2006). Variables measured in yellow channels included spot coordinates, spot area and spot density, which were used to calculate total number of spots and total spot density. These were further normalized per the number of cells in each image for the experimental groups to give values for the "number of spots per cell" and the average "density per spot," as shown in the graphs.

Discrete spots were generally not observed in CA3, so a semi-quantitative measure of the colocalization of ubiquitin with either S6 or TIA-1 was achieved by taking the integrated density of the entire yellow channel, normalized to the number of cells in each image to produce a value of "density per cell." These measurements were made using NIH ImageJ (Abramoff et al., 2004).

All quantitative data from the experimental groups were compared by ANOVA followed by LSD post hoc with statistical significance set at P<0.05.

Microdissection of CA1 and CA3

The above experimental groups were repeated (n=4 per group). Following the respective durations of reperfusion, animals were killed with 5% halothane, decapitated, and brains dissected and snap frozen in dry ice and ethanol. Hippocampal CA1 and CA3 were dissected, under a dissecting microscope, from semi-frozen slices obtained from a coronal section of the brain cut approximately −2.30 mm to −3.80 mm posterior to Bregma (Paxinos and Watson, 1998). The CA3 region was separated by a vertical cut slightly medial to the curve of the CA3 followed by a second vertical cut at the lateral edge of the dorsal hippocampus. Surrounding cortex was removed to provide isolated CA3. The CA1 region was separated from the DG by a roughly horizontal cut passing through the obliterated hippocampal fissure which is delineated by a continuous line of large, cross-sectioned blood vessels. The corpus callosum on the superior surface was removed to complete the isolation of CA1. The resulting tissue dissections were weighed and then hand-homogenized on ice and further processed as described below. For a single rat, bilateral dissections of CA1 and CA3 gave 3–7 mg wet weight per each region.