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PKR-LIKE ENDOPLASMIC RETICULUM KINASE (PERK) ACTIVATION FOLLOWING BRAIN ISCHEMIA IS INDEPENDENT OF UNFOLDED NASCENT PROTEINS

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Abstract—Transient global brain ischemia results in an immediate inhibition of protein translation upon reperfusion. During early brain reperfusion protein synthesis is inhibited by alpha subunit of eukaryotic initiation factor 2 (eIF2α) phosphorylation by the PKR-like endoplasmic reticulum kinase (PERK). Normally, PERK is held in an inactive, monomeric state by the binding of the endoplasmic reticulum (ER) chaperone GRP78 to the lumenal end of PERK. The prevailing view is that ER stress leads to the accumulation of unfolded proteins in the ER lumen. GRP78 dissociates from PERK to bind these accumulated unfolded proteins, leading to PERK activation, phosphorylation of eIF2α, and inhibition of translation. To determine if an increase in unfolded nascent proteins following transient brain ischemia contributes to PERK activation, protein synthesis was blocked by intracerebral injection of anisomycin prior to induction of ischemia. Anisomycin inhibited protein synthesis by over 99% and reduced newly synthesized proteins in the ER to ~20% of controls. With an ER nearly devoid of newly synthesized proteins, PERK was still activated and was able to phosphorylate eIF2α in CA1 neurons during reperfusion. These data strongly argue that PERK activation is independent of the large increase in unfolded nascent proteins within the ER following transient global brain ischemia. Published by Elsevier Ltd on behalf of IBRO.

Key words: brain, ischemia, PERK, eIF2, UPR, ER stress.

Transient global cerebral ischemia induces inhibition of protein synthesis in neurons throughout the entire brain (Hossmann, 1993; Krause and Tiffany, 1993). In resilient regions of the brain, neurons eventually re-establish protein production and homeostasis is restored. However, in the region most vulnerable to transient ischemia, the CA1 region of the hippocampus, protein synthesis never recovers (Araki et al., 1990; Thilmann et al., 1986). Prolonged absence of protein synthesis is clearly detrimental and a number of investigators have suggested that continued inhibition of protein synthesis in vulnerable neurons contributes to death of these cells during brain reperfusion (Nakagomi et al., 1993; Paschen, 2000; White et al., 2000).

Previously, we reported that inhibited translation initiation is responsible for the suppression of protein synthesis in the reperfused brain (DeGracia et al., 1996; Sullivan et al., 1999). The overall rate of protein synthesis is decreased by phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α), which can be caused by wide variety of cellular stresses (Bröstrom and Bröstrom, 1998). Immediately upon reperfusion following complete global ischemia, there is a 20-fold increase in phosphorylated eIF2α [eIF2α(P)] in neurons throughout the brain (Kumar et al., 2001). These elevated levels of eIF2α(P) result in near-complete translation inhibition during early reperfusion in neurons throughout the brain (Sullivan et al., 1999).

Four kinases are known to phosphorylate eIF2α, however, we have shown the PKR-like endoplasmic reticulum kinase (PERK) is solely responsible for the large increase in eIF2α(P) during early reperfusion (Owen et al., 2005). PERK is a transmembrane endoplasmic reticulum (ER) stress-sensor protein that, along with Ire1 and ATF6, constitute the proximal components of an ER stress response generally referred to as the unfolded protein response (UPR).

The prevailing UPR model asserts that ER stress disrupts the protein processing machinery leading to accumulation of unfolded proteins in the ER which causes dissociation of the chaperone GRP78 from the lumenal ends of PERK, Ire1, and ATF6 (Zhang and Kaufman, 2006; Wek and Cavener, 2007; Ron and Walter, 2007). GRP78 is thought to dissociate from these ER stress sensors and bind to unfolded proteins, thereby preventing aggregation and improper folding. GRP78 dissociation from PERK has been observed following brain ischemia (Hayashi et al., 2003). Importantly, the stimulus that causes GRP78 dissociation following brain ischemia has not been identified. Typically, initiation of the UPR involves activation of all three ER stress sensor proteins, PERK, Ire1, and ATF6. However, following ischemia the canonical UPR is not executed. Instead, of the three ER stress sensors, only PERK is activated during early brain reperfusion (Kumar et al., 2003) suggesting that PERK, Ire1 and ATF6 may not be activated by a shared mechanism following global brain ischemia. Previous studies showed that when ER were

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Abbreviations: DPM, disintegration per minute; eIF2α, alpha subunit of eukaryotic initiation factor 2; ER, endoplasmic reticulum; PERK, PKR-like endoplasmic reticulum kinase; UPR, unfolded protein response.

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experimentally loaded with an unfolded protein there was activation of the UPR (Mori et al., 1992; Puig and Gilbert, 1994). This lead to an intuitive hypothesis that unfolded proteins were in fact the direct stimulus for UPR activation. However, it has never been directly demonstrated that generic unfolding of transiting proteins causes PERK activation under any stress conditions.

To test the hypothesis that an increase in the unfolded protein load was the proximate cause of PERK activation following brain ischemia, we asked the converse question: in the absence of an unfolded protein load, would PERK fail to be activated by an ischemic insult? We experimentally blocked new protein synthesis and significantly cleared the ER of transiting proteins prior to the onset of ischemia. We then assayed PERK phosphorylation and PERK's ability to phosphorylate eIF2α during reperfusion and found that, even in the absence of unfolded proteins, PERK was activated.

EXPERIMENTAL PROCEDURES

Anisomycin injection

Male Long Evans rats weighing 350 to 400 g were anesthetized with halothane (Henry Schein, Inc., Melville, NY, USA), orotracheally intubated, and mechanically ventilated. Rectal temperature was maintained at 37.0±0.5 °C. Cannulae were positioned bilaterally in the CA1 region of the dorsal hippocampi using a stereotaxic apparatus (4.5 mm posterior to bregma; 3.0 mm lateral to the midline; and 3.5 mm ventral to the skull surface). Ninety-minutes prior to induction of ischemia, 5.0 μl of either the protein synthesis inhibitor anisomycin (250 μg; Sigma) or vehicle (4% Methylene Blue in 75% phosphate-buffered saline/25% dimethyl sulfoxide, pH 7.5) was infused through the cannulae over 30 s through a 27-gauge catheter. Anisomycin blocks protein synthesis by interfering with the peptidyl transferase activity of the large ribosomal subunit. The catheter was left in place for an additional 2 min to permit diffusion of the drug. Successful delivery of anisomycin to the intended target site was verified by including Methylene Blue in all injection solutions; the presence of Methylene Blue in hippocampal tissue was used as an indicator of the diffusion of anisomycin. Infusions were considered correct if the spread of the dye was within 1 mm^3 of the intended site (Martin, 1991), and only the dye-stained areas were used in these studies. All animal procedures were in compliance with the National Institutes of Health guidelines for ethical use of animals and were approved by the Wayne State University Animal Investigation Committee.

Animal model

Global brain ischemia of 8 min duration was induced using bilateral carotid occlusion and hypotension as described by Smith et al. (1984) and modified by Sanderson et al. (2008). A femoral artery catheter was inserted and arterial blood pressure was continually monitored. A midline incision was made in the ventral neck, and the carotids were bluntly dissected. Ischemia was induced by rapidly withdrawing blood from the femoral artery to achieve a mean arterial pressure of 31–35 mm Hg (Sugawara et al., 2000). Both carotids were then occluded with microaneurysm clips. After 8 min, the clips were removed and the withdrawn blood, maintained at 37 °C, was reinfused to achieve a mean arterial pressure of 70–90 mm Hg within 2 min. This model consistently causes ~95% loss of pyramidal neurons in the CA1 hippocampus (Sanderson et al., 2008).

After 10 min of reperfusion, animals were transcardially perfused with ice-cold isotonic saline, and the brain was rapidly removed. For Western blot analysis, the hippocampal isolation began with a midline incision along the longitudinal fissure through the corpus callosum followed by careful separation of the telencephalon from the diencephalon. The hippocampus was then rapidly dissected away and the entire dye-stained area was collected regardless of hippocampal sub-field because early PERK activation is ubiquitous throughout the entire hippocampus during early brain reperfusion. When brains were used for immunofluorescence, the rats were transcardially perfused with 4% paraformaldehyde, postfixed for an additional 2 h, cryoprotected in 20% then 30% sucrose in PBS, and then frozen in isopentane and dry ice.

In vitro translation

In vitro translation was performed by a modification of the method of Cosgrove and Rapoport (1986), as we have previously described (DeGracia et al., 1996), using brain homogenates prepared from the dye-stained area. To obtain the post-mitochondrial supernatant (PMS), hippocampal tissue was weighed, Dounce homogenized in 1:2.5 (wt:vol) isotonic buffer (50 mM HEPES, pH 7.54, 140 mM potassium acetate, 4 mM magnesium acetate, 2.5 mM DTT, 10 μg/mL aprotinin, 4 μg/mL leupeptin, 4 μg/mL pepstatin A) and then centrifuged at 10,000×*g* for 10 min at 4 °C. The final supernatant was collected and the protein concentration was determined by the Lowry method. Translation reactions consisted of 375 μg hippocampal protein, 100 μM each amino acid except methionine and cysteine, and 1.5 μCi [^{35}S]-methionine/cysteine ([^{35}S]-met/cys, specific activity 1175 Ci/mmol) in a final volume of 225 μl and were run for 12 min.

Sub-cellular fractionation

The dye-stained region was microdissected from the isolated hippocampi. The hippocampi were separated into microsomal (enriched endoplasmic reticulum) and heavy membrane (enriched plasma membrane) fractions using differential centrifugation. Hippocampi were first homogenized in 1:10 (wt:vol) fractionation buffer (250 mM sucrose, 1 mM HEPES-KOH pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.5 mM DTT, 1 mM EGTA, 10 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mM PMSF) using 15 strokes with a Potter-Elvejhem tissue homogenizer with a loosely fitting pestle (0.2-mm clearance, 150 rpm, 4 °C). Homogenates were centrifuged in an Avanti J-E centrifuge (Beckman Coulter, Fullerton, CA, USA) at 1,000×*g* for 10 min. The pellets were resuspended in 1:10 fractionation buffer, re-homogenized and centrifuged again at 1,000×*g* for 10 min. Combined supernatants were centrifuged at 20,000×*g* for 20 min and the resulting pellet was taken as the heavy membrane fraction. The supernatant was centrifuged for 1 h at 140,000×*g* in a LE-80K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) and the resulting pellet was taken as the microsomal fraction. Purity of the preparation was confirmed by Western blot using antibodies against an ER marker (ERp29) and a plasma membrane marker (pan cadherin).

In vivo protein labeling

Rats were anesthetized as described above. A femoral venous line was inserted and a 1 mCi bolus of [^{35}S]-met/cys was administered i.v. 30 min prior to injection of either anisomycin or vehicle into the hippocampus to allow complete labeling of nascent proteins. After 90 min of anisomycin or vehicle incubation, the animals were sacrificed and the hippocampi were microdissected (Fig. 1B). The dye-stained area was excised, homogenized in fractionation buffer and separated as described in subcellular fractionation. Protein concentrations were determined using the Lowry method. Equal protein amounts (25 μg) were spotted on