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HYPOXIC PRECONDITIONING AND CELL DEATH FROM OXYGEN/GLUCOSE DEPRIVATION CO-OPT A SUBSET OF THE UNFOLDED PROTEIN RESPONSE IN HIPPOCAMPAL NEURONS

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Abstract—The state of protein folding in the endoplasmic reticulum (ER), via the unfolded protein response (UPR), regulates a pro- or anti-apoptotic cell fate. Hypoxic preconditioning (HPC) is a potent anti-apoptotic stimulus, wherein ischemic neural injury is averted by a non-damaging exposure to hypoxia. We tested if UPR modulation contributes to the pro-survival/anti-apoptotic phenotype in neurons preconditioned with hypoxia, using organotypic cultures of rat hippocampus as a model system. Pharmacologic induction of the UPR with tunicamycin increased mRNA of 79 of 84 UPR genes and replicated the pro-survival phenotype of HPC, whereas only small numbers of the same mRNAs were upregulated at 0, 6 and 24 h after HPC. During the first 24 h after HPC, protein signals in all 3 UPR pathways increased at various times: increased ATF4, phosphorylation of eif2α and IRE1, cleavage of *xbb1* mRNA and cleavage of ATF6. Pharmacologic inhibition of ATF6 and IRE1 blocked HPC. Ischemia-like conditions (oxygen/glucose deprivation, OGD) caused extensive neuron cell damage and involved some of the same UPR protein signals as HPC. In distinction to HPC and tunicamycin, OGD caused widespread suppression of UPR genes: 55 of 84 UPR gene mRNAs were numerically downregulated. We conclude that although HPC and ischemic cell death in hippocampal neurons involve protein-based signaling in all 3 UPR pathways, these processes co-opt only a subset of the genomic response elicited by agents known to cause protein misfolding, possibly because of persistent transcription/translation arrest induced by hypoxia and especially OGD.
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Key words: hypoxic preconditioning, hippocampal slice cultures, cerebral ischemia, neuroprotection.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; GF-EBSS, glucose-free Earle's Basic Salt Solution; HPC, hypoxic preconditioning; HSCs, hippocampal slice cultures; OGD, oxygen and glucose deprivation; PI, propidium iodide; RT, Reverse Transcriptase; Xbp1, X-box protein 1.

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INTRODUCTION

Transient global ischemia causes selective neuronal death in area CA1 of the hippocampus, resulting in impairment of cognition and memory (Volpe and Petito, 1985). Preconditioning with a non-injurious period of ischemia or hypoxia (HPC) induces an ischemia-resistant phenotype in the hippocampus and other brain regions that enables cells to avoid apoptosis after stress (Kitagawa, 2012). The mechanisms of HPC have been extensively studied but it has not been possible to unequivocally identify the most important events in the acquisition of an ischemia-resistant phenotype (Simon et al., 2007; Shpargel et al., 2008; Lehotsky et al., 2009). Furthermore, preconditioning has not been applied clinically to reduce ischemic brain injuries.

Regulation of protein homeostasis by the endoplasmic reticulum (ER) appears to be involved in both preconditioning (Mao and Crowder, 2010) and ischemia-induced cell death (Paschen et al., 2003; DeGracia and Montie, 2004; Paschen and Mengesdorf, 2005). Proteomic stress is also involved in neurodegenerative conditions such as Parkinson's disease (Valdes et al., 2014). Assessed by accumulation of polyubiquitinated proteins, the post-ischemic brain experiences substantial proteomic dysfunction (Hu et al., 2000, 2001; Liu et al., 2005a,b; Zhang et al., 2006; Iwabuchi et al., 2014). During hypoxic or ischemic stress, the UPR detects misfolded secretory proteins in the ER lumen and re-establishes homeostasis, possibly contributing to the ischemia-resistant phenotype of preconditioned cells. If post-ischemic protein misfolding is overwhelming, the UPR initiates apoptosis via ER caspase-12 (Nakka et al., 2010), the transcription factor, C/EBP homologous protein (CHOP) (Tajiri et al., 2004), and the apoptosis signal-regulating kinase-1 (ASK-1) (Nishitoh et al., 2002).

Previous studies on mammalian neurons have not determined whether all 3 UPR pathways are equally important in determining cell fate following ischemia-like stress (Badiola et al., 2011; Sanderson et al., 2015). The question of pathway priority is complex, because stress signaling involves many overlapping pathways and processes in addition to those within the canonical UPR. For example, the integrated stress response pathway involving MAPK and JNK cascades regulates the PERK pathway protein eif2α (Thiaville et al., 2008), a convergent effector in stress responses (Wek et al., 2006; Sidrauski et al., 2013). To help resolve this issue, we used

a well-characterized hippocampal slice culture (HSC) model and a number of approaches designed to reveal the role of the different arms of the UPR at various time points following hypoxic and ischemia-like events. We examined hypoxia, in durations producing ischemic tolerance and hypoxic injury, and simulated ischemia with oxygen and glucose deprivation (OGD). Because signaling responses to hypoxia in the hippocampus vary temporally (Bickler and Fahlman, 2009; Bickler et al., 2010), we assessed UPR pathway signals immediately after, 6 h after and 24 h after hypoxia or simulated ischemia. Examination of signals during the period of latency between preconditioning and tolerance has rarely been done. We sought evidence for proteomic stress by assessing protein polyubiquitination and caspase-12 activation. To assess the status of each of the three arms of the UPR (ATF6, PERK and IRE1 pathways) we employed protein expression and phosphorylation analysis, cleavage of X-box protein 1 (Xbp1) mRNA and UPR gene mRNA using expression arrays. Tunicamycin, an antibiotic that interferes with protein folding by inhibiting glycosylation, was used as a positive control for UPR expression. In addition, using pharmacologic tools, we assessed the survival role of the ATF6 and IRE1 pathways in producing the ischemia tolerant phenotype in hippocampal neurons.

EXPERIMENTAL PROCEDURES

Preparation of HSCs

HSCs were prepared with standard methods (Stoppini et al., 1991), with key procedures as follows: Post-natal day (P7) Sprague–Dawley rats (Charles River Laboratories, Hollister, CA, USA) were decapitated and the hippocampi were quickly removed and placed in chilled Gey's balanced salt solution (UCSF cell culture facility) containing 25 mM HEPES, 6.5 mg/ml D-glucose, and 100 mM sucrose. The hippocampi were sliced 400 μm thick using a McIlwain tissue chopper as shown in a video (Mitchell et al., 2010). The slices were placed on 30-mm diameter Millipore membrane inserts (Millicell PICM03050, Millipore, Billerica, MA, USA) in six-well culture trays, 4–5 slices per well with 1.2 ml culture media, and placed in an air/CO_2 incubator at 37 °C. The slice culture medium for the first week *in vitro* consisted of BME media, 23% heat-inactivated horse serum, 1 mM GlutaMax, 9 mM D-glucose and 1× penicillin/streptomycin. A humidified atmosphere of 5% CO_2 and 95% air at 37 °C was used. The media was replaced on the day after slicing and then every other day. On day 7 the media was changed to neurobasal media with 2% B-27, 1 mM GlutaMax, 0.5 mM ascorbic acid, 9 mM D-glucose and 1× penicillin/streptomycin. This media was replaced every other day through day 14. On day 14 the media was replaced with neurobasal with 2 mM L-glutamine. On day 16, the slices were randomized to receive further treatments. Media and reagents were from the UCSF cell culture facility, Life Technologies (Thermo Fisher Scientific, Grand Island, NY, USA) or Sigma (Sigma Aldrich, St. Louis, MO, USA).

Approximately 16 slices were harvested from each of the ~300 animals sacrificed for the study. Slices were pre-selected so that only those with normal morphology in the CA1, CA3, and dentate neuron cell body regions were cultured. Prior to the experiments, the plates were shuffled and selected at random for each treatment group to ensure that every group contained cultures from multiple animals. This process also ensured a random sample of slices from both male and female rat pups in each treatment group.

Hypoxia, hypoxic preconditioning (HPC), and simulated ischemia

For preconditioning or damaging hypoxia without ischemia-like conditions, Billups–Rothenberg chambers ("BR" chambers, Billups-Rothenburg Incorporated, Del Mar, CA, USA) containing the culture dishes were flushed with warmed and humidified 95% N_2/5% CO_2. This flush, done to remove ambient oxygen, was continued for 5 min. The sealed BR chambers were then placed in 37 °C incubators. The study design for preconditioning involved 5–30 min of hypoxia and more stressful durations of 60 min and 120 min of hypoxia were also studied. Fifteen–thirty minutes of hypoxia induces the ischemia- tolerant phenotype, whereas 60 min or 120 min of hypoxia typically causes damage or increases the sensitivity of the slice cultures to damage caused by OGD. Following treatment, slices were removed from the BR chambers and returned to standard media in an air/5% CO_2 incubator.

To expose HSCs to ischemia-like conditions (OGD), warmed glucose-free Earle's Basic Salt Solution (GF-EBSS) was bubbled with 95% N_2/5% CO_2 for 15 min to remove all oxygen. Cultures were rinsed in GF-EBSS then placed in culture trays containing GF-EBSS and placed in BR chambers without the culture tray lids. The cultures were left in the incubator for 24 h before being exposed to either 1-h OGD, which causes moderate damage, or 2 h of OGD, which kills the vast majority of neurons in the cultures. The temperature of the media was 37 °C, measured with a thermocouple thermometer. The partial pressure of oxygen, measured with a polarographic oxygen electrode, was 0–0.2 mm Hg. After this insult, the cultures were returned to standard slice culture media at 37 °C. The percentages of dead and living neurons in CA1, CA3 and dentate were measured 24 h later.

The expression of genes in the hippocampus of rats after hypoxia or ischemia follows a changing temporal pattern over a 24-h period (Bickler and Fahlman, 2009). Therefore, we assessed UPR protein expression and mRNA levels both immediately and 6 and 24 h after completion of preconditioning hypoxia. In addition, we assessed mRNA coding for UPR genes 24 h after OGD.

To induce an ER stress response, slices were exposed to 10 μg/mL tunicamycin from *Streptomyces* sp. (Sigma) for 24 h. The slices were then returned to standard media and 24 h later were harvested for protein or mRNA expression analysis. That time point was chosen based on preliminary studies showing full UPR gene expression but concentration-dependent cell death at 24 h.

UNCORRECTED PROOF

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