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TEMPORAL DEPENDENCE OF CYSTEINE PROTEASE ACTIVATION FOLLOWING EXCITOTOXIC HIPPOCAMPAL INJURY

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Abstract—Excitotoxic insults can lead to intracellular signaling cascades that contribute to cell death, in part by activation of proteases, phospholipases, and endonucleases. Cysteine proteases, such as calpains, are calcium (Ca^{2+})-activated enzymes which degrade cytoskeletal proteins, including microtubule-associated proteins, tubulin, and spectrin, among others. The current study used the organotypic hippocampal slice culture model to examine whether pharmacologic inhibition of cysteine protease activity inhibits its *N*-methyl-D-aspartate- (NMDA-) induced excitotoxic (20 μM NMDA) cell death and changes in synaptophysin immunoreactivity. Significant NMDA-induced cytotoxicity (as measured by propidium iodide [PI] uptake) was found in the CA1 region of the hippocampus at all timepoints examined (24, 72, 120 h), an effect significantly attenuated by co-exposure to the selective NMDA receptor antagonist DL-2-Amino-5-phosphonopentanoic acid (APV), but not MDL-28170, a potent cysteine protease inhibitor. Results indicated sparing of NMDA-induced loss of the synaptic vesicular protein synaptophysin in all regions of the hippocampus by MDL-28170, though only at early timepoints after injury. These results suggest Ca^{2+}-dependent recruitment of cysteine proteases within 24 h of excitotoxic insult, but activation of alternative cellular degrading mechanisms after 24 h. Further, these data suggest that synaptophysin may be a substrate for calpains and related proteases.
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Key words: NMDA receptors, excitotoxicity, synaptophysin, cysteine protease, calpain.

INTRODUCTION

Excitotoxicity is the overexcitation of neurons due to the excessive activation of excitatory amino acid (EAA) receptors and likely contributes to central nervous system injury in neurodegenerative states, including traumatic brain injury (TBI), ischemia, stroke, epilepsy, and amyotrophic lateral sclerosis (see Choi, 1992 for a review). Calcium (Ca^{2+})-permeable glutamatergic *N*-methyl-D-aspartate (NMDA) receptors are thought to be an integral component of the excitotoxic cascade. For example, Choi et al. reported that co-exposure to competitive NMDA receptor antagonists attenuated NMDA- and glutamate-excitotoxicity in primary cortical cell cultures (Choi et al., 1988), while antagonists of AMPA or kainate-type glutamate receptors (GluRs) did not. Neurons maintain low intracellular Ca^{2+} concentrations, as compared to extracellular space, by regulating Ca^{2+} efflux, intracellular Ca^{2+} buffering, and intracellular Ca^{2+} storage (Sattler and Tymianski, 2000). Although small, physiologically relevant amounts of Ca^{2+} are necessary for the cell to function properly, excessive Ca^{2+} influx, such as that seen during a state of neural hyperexcitability, may quench regulatory mechanisms and initiate cytotoxicity associated with activity of proteases, phospholipases, and endonucleases (Choi, 1995; Sattler and Tymianski, 2000) or alterations in mitochondrial bioenergetics (for a review, see Pivovarova and Andrews, 2010).

Cysteine proteases, such as calpains, are Ca^{2+}-activated enzymes that degrade cytoskeletal proteins, including microtubule-associated proteins, tubulin, and spectrin, among others (Siman and Noszek, 1988; Vosler et al., 2008). Siman and colleagues (1989) reported that EAA-induced calpain I activation is closely associated with EAA-induced hippocampal damage since only those doses of intraventricular-administrated EAAs which produce hippocampal damage also resulted in calpain I activation. Further, calpain inhibition with use of the cysteine protease inhibitor MDL-28170 was found to be neuroprotective when given either as a pretreatment or immediately following a glutamate challenge in primary hippocampal cultures (Rami et al., 1997). Gellerman et al. (1997) reported that loss of GluR1 subunits in the hippocampus following NMDA exposure was prevented by co-exposure to calpain inhibitor I or calpeptin. One recent study reported that both NMDA and non-NMDA GluR agonism activated calpain as reflected in accumulation of spectrin breakdown products; however, only the NMDA receptor antagonist was effective at reducing glutamate-induced toxicity (Del Rio et al., 2008). These breakdown products have been detected *in vitro* following as little as a 5-min exposure to NMDA (Bahr et al., 1995), while *in vivo* work has suggested that

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Abbreviations: AMPAr, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor; ANOVA, analysis of variance; APV, DL-2-amino-5-phosphonopentanoic acid; CA, cornu ammonis; Ca^{2+}, calcium; DG, dentate gyrus; DMSO, dimethyl sulfoxide; EAA, excitatory amino acid; endoG, endonuclease G; FITC, fluorescein isothiocyanate; GluR, glutamate receptor; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PI, propidium iodide; TBI, traumatic brain injury.

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spectrin breakdown takes hours or days to be observed following NMDA-induced neurodegeneration (Siman et al., 1989). Previous research has also indicated that calpain-dependent axonal varicosities appear prior to neuronal damage following glutamate-induced excitotoxic insult (Hou et al., 2009). Thus, there has been confusion regarding which markers might reflect calpain-mediated cell death and the timecourse associated with the expression of these markers remains unclear. Vosler et al. (2008) describe evidence for the existence of more than sixty calpain substrates in neurodegeneration.

Several notable constituents of the cytoskeleton, including the vesicle protein synaptophysin, are not clearly defined as calpain substrates. Research examining the possibility that synaptophysin may be a substrate for calpain and related proteases has yielded contradictory results (Thompson et al., 2006; Lee et al., 2008). Synaptophysin is a synaptic vesicle protein found in the axon terminal of presynaptic neurons and has been shown to be present in the brain and spinal cord (Wiedenmann and Franke, 1985). Synaptophysin is thought to be involved in the docking and fusion of the vesicle with the plasma membrane (Sudhof, 1995) and has been implicated in synaptogenesis and synaptic reorganization (Bergmann et al., 1997). Accordingly, synaptophysin has been used to assess the integrity of axon terminals in a number of injury paradigms, including glutamate-induced excitotoxicity (Lee et al., 2008), TBI (Thompson et al., 2006), and ischemia (Stroemer et al., 1995).

The present studies employed the organotypic hippocampal cell culture model to examine the temporal and topographical nature of NMDA exposure and cysteine protease activity on neuronal viability and synaptophysin abundance. The organotypic model has been shown to closely mimic *in vivo* structural and functional integrity over time and serves as a sensitive model of NMDA receptor-mediated effects on neuronal viability (Gutierrez and Heinemann, 1999; Martens and Wree, 2001). The temporal and topographical characterization of cellular injury or death following EAA receptor activation may be particularly important in further identifying novel substrates, such as synaptophysin, for calpain and other cysteine proteases in excitotoxic neurodegeneration. Further, it was hypothesized that the effects associated with NMDA receptor overactivation will be more pronounced in the cornu ammonis 1 (CA1) region of the hippocampus in accordance with previous reports (Butler et al., 2010).

EXPERIMENTAL PROCEDURES

Organotypic hippocampal slice culture procedure

Eight-day-old male and female Spraque–Dawley rats (acquired from Harlan Laboratories; Indianapolis, IN, USA) were humanely sacrificed and the brains were aseptically removed. Following removal, brains were transferred into ice-cold dissecting media, composed of Minimum Essential Medium (Invitrogen, Carlsbad, CA, USA), 25 mM HEPES (Sigma, St. Louis, MO, USA), and 50 μM streptomycin/penicillin (Invitrogen). Bilateral hippocampi were removed in whole and cleaned of extra tissue under a dissecting microscope. Hippocampi were then placed into culture media, which contain dissecting medium along with distilled water, 36 mM glucose (Fisher, Pittsburgh, PA, USA), 25% Hanks' Balanced Salt Solution (Invitrogen), 25% (v/v) heat-inactivated horse serum (Sigma), and 0.05% streptomycin/penicillin. Unilateral hippocampi were sectioned at 200 μm using a McIllwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Following sectioning, three intact hippocampal slices were plated onto a Millicell-CM 0.4-μm biopore membrane insert with 1 mL of pre-incubated culture media added to the bottom of each well of a six-well plate, yielding a total of 18 intact slices per plate. Excess culture medium was aspirated from the top of each well and the plates were then incubated at 37 °C with a gas composition of 5% CO_2/95% air for 5 days to allow tissue to affix to the Teflon membrane. Care of all animals was carried out in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and the University of Kentucky's Institutional Animal Care and Use Committee.

Drug exposure

At 5 days *in vitro*, slices were randomly transferred to new culture plates containing either 1 mL of culture media containing propidium iodide (PI; 3.74 μM; Molecular Probes, Eugene, OR, USA) or 1 mL of culture media containing PI and the following drugs: NMDA (20 μM; Sigma); MDL-28170 (2.5 or 25 μM; Sigma); NMDA + MDL-28170 (as above); DL-2-Amino-5-phosphonopentanoic acid (APV) (50 μM; Sigma); or NMDA + APV (as above) dissolved in 1 mL of culture media. MDL-28170 was first dissolved in dimethyl sulfoxide (DMSO; Fisher) to yield a final concentration of 0.5% DMSO in culture media. Thus, an additional group of slices was exposed to only 0.5% DMSO. For studies involving MDL-28170, cultures were first exposed to MDL-28170 or culture media 2 h prior to the addition of NMDA. PI is a nucleic acid stain used to detect cell damage and was used to measure cytotoxicity as described below. All experiments were replicated at least twice, with each experimental condition (treatment × exposure time × sex within region) containing $n = 18–27$ slices. A total of 14 rat litters were used for these experiments.

Cytotoxicity assessment

Previous work has shown that PI reliably correlates with other measures of cell death (for a review, see Zimmer et al., 2000), and as such was used as a measure of cytotoxicity in the present studies (i.e. staining of neurons and glia with compromised membranes). PI was measured in the granule cell layer of the dentate gyrus (DG) as well as in the pyramidal cell layers of the cornu ammonis 3 (CA3) and the CA1 regions of the hippocampal formation using fluorescent microscopy. Slices were visualized with SPOT advanced version 4.0.2 software for Windows (W. Nuhsbahm Inc.; McHenry, IL, USA) using a 5x objective with a Leica DMIRB microscope (W. Nuhsbahm Inc.; McHenry, IL, USA) fitted for fluorescence detection (mercury-arc lamp) using blue-green light and connected to a personal computer through a SPOT 7.2 color mosaic camera (W. Nuhsburg Inc.; McHenry, IL, USA). PI has an emission wavelength of 620 nm in the visual range and a peak excitation wavelength of 536 nm and was excited using a band-pass filter which excites a range of wavelengths (510–560 nm). Densitometry using Image J software (National Institutes of Health, Bethesda, MD, USA) was used to measure the intensity of PI fluorescence within each hippocampal region. A background measurement was taken from the visual field surrounding each slice and was subsequently subtracted from the regional measurement from each slice. Within each region,

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