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Brief communication

Pharmacological blockade of the calcium plateau provides neuroprotection following organophosphate paraoxon induced status epilepticus in rats

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ABSTRACT

Organophosphate (OP) compounds which include nerve agents and pesticides are considered chemical threat agents. Currently approved antidotes are crucial in limiting OP mediated acute mortality. However, survivors of lethal OP exposure exhibit delayed neuronal injury and chronic behavioral morbidities. In this study, we investigated neuroprotective capabilities of dantrolene and carisbamate in a rat survival model of paraoxon (POX) induced status epilepticus (SE). Significant elevations in hippocampal calcium levels were observed 48-h post POX SE survival, and treatment with dantrolene (10 mg/kg, *i.m.*) and carisbamate (90 mg/kg, *i.m.*) lowered these protracted calcium elevations. POX SE induced delayed neuronal injury as characterized by Fluoro Jade C labeling was observed in critical brain areas including the dentate gyrus, parietal cortex, amygdala, and thalamus. Dantrolene and carisbamate treatment provided significant neuroprotection against delayed neuronal damage in these brain regions when administered one-hour after POX-SE. These results indicate that dantrolene or carisbamate could be effective adjuvant therapies to the existing countermeasures to reduce neuronal injury and behavioral morbidities post OP SE survival.

1. Introduction

Organophosphate (OP) compounds are classified as lethal chemicals that include nerve gas and pesticides. Both the civilian and military population has been exposed to nerve agents under acts of war and terrorism (Haley and Tuite, 2013; Hood, 2001; Sellstrom et al., 2013). In addition, civilians are also exposed to OP compounds occupationally, intentionally (suicide) or due to accidents (Ajdacic-Gross et al., 2008; Konradsen, 2007; Than, 2013). Paraoxon (POX) is an active metabolite of parathion and is used in laboratory research to reliably model OP pesticide toxicity (Deshpande et al., 2014a). POX and other OP chemicals are potent inhibitors of the enzyme acetylcholine esterase (AChE) (Tuovinen, 2004). Inhibition of AChE prevents breakdown of acetylcholine (ACh) and rapidly builds up its level at the synapses. Overt stimulation of ACh receptors leads to the classical "cholinergic crisis" followed by respiratory depression, bradycardia and status epilepticus (SE). This prolonged seizure activity represents a clinical emergency and if left untreated results in the death (Bajgar, 2004). The current FDA approved OP treatment protocol involves administration of an anticholinergic drug atropine to manage hypercholinergic symptoms, an oxime pralidoxime to reactivate AChE, and a benzodiazepine midazolam to stop SE (Chemical Hazards Emergency Medical Management, 2013). Despite the effectiveness of the standard three-drug regimen in limiting immediate mortality following OP exposure, OP/SE survivors are vulnerable to the development of chronic neurological morbidities (de Araujo Furtado et al., 2012; Deshpande et al., 2014b; Helmstaedter, 2007; Neligan and Shorvon, 2011; Rod, 2009; Phillips and Deshpande, 2016; Savage et al., 1988).

Our laboratory has developed SE survival models of OP toxicity using POX (Deshpande et al., 2014a; Deshpande et al., 2014b) and DFP (Deshpande et al., 2010). The mortality, behavioral manifestations and EEG profile for these OP SE models mimicked the signs and symptoms of human OP intoxication. Significant neuronal damage was observed throughout the limbic system in the brain of OP SE rats (Deshpande et al., 2014a; Deshpande et al., 2010). Subsequently, symptoms of chronic depression and memory impairments were also observed in these lethal OP exposed rats (Deshpande et al., 2014a; Deshpande et al., 2014b). These models provide a reproducible method to mimic the human survival of OP toxicity and are useful to screen novel medical countermeasures and also identify molecular mechanisms underlying the mortality and morbidity following OP intoxication.

One of the long standing interests of our laboratory has been studying role of Ca^{2+} homeostatic mechanisms following brain injuries (DeLorenzo et al., 2005). Ca^{2+} ions are major second messenger

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molecules and they participate in a variety of signaling cascades critical for learning, memory, neuronal injury and other vital cellular functions. We have demonstrated significant elevations in neuronal calcium levels ($[Ca^{2+}]_i$) that lasted for weeks following the termination of brain injuries, such as SE, stroke or TBI. These protracted elevations in $[Ca^{2+}]_i$ known as the Ca^{2+} plateau (Deshpande et al., 2014a; Deshpande et al., 2010; Raza et al., 2004; Nagarkatti et al., 2010; Filbert et al., 2005) could underlie the neuronal injury and the chronic neurological morbidities following survival from OP SE. Interestingly, Ca^{2+} influx from NMDA receptors was required for induction of this Ca^{2+} plateau (Deshpande et al., 2010), but the protracted Ca^{2+} elevations were mediated by intracellular Ca^{2+} induced Ca^{2+} release (CICR) from endoplasmic reticulum. Indeed, we have recently shown that treatments with the CICR antagonist dantrolene, and carisbamate, but not the NMDA antagonist MK-801 lowered OP-SE mediated Ca^{2+} elevations (Deshpande et al., 2014a; Nagarkatti et al., 2010). In this study, we investigated whether inhibition of this Ca^{2+} plateau with dantrolene and carisbamate could provide neuroprotection when administered after the termination of OP SE. Given the role of Ca^{2+} signaling in modulating behavior and cell death mechanisms (Bengtson and Bading, 2012; Baker et al., 2013), preventing the development of Ca^{2+} plateau could be a critical target to improve outcome following lethal OP exposure.

2. Materials and methods

2.1. Animals

All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by VCU's Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing ~250–280 g and 10-weeks age were housed two per cage at 20–22 °C with a 12 Light: 12 Dark hour cycle and free access to food and water.

2.2. Chemicals

All the chemicals were obtained from Sigma Aldrich Company (St. Louis, MO). POX was prepared by dissolving in ice-cold phosphate buffered saline, while atropine sulfate and pralidoxime chloride (2-PAM) were dissolved in saline (0.9% NaCl). Midazolam was obtained from VCU Health System Pharmacy. Carisbamate (RWJ 333369) was a gift from Johnson & Johnson PRD (Titusville, NJ, USA), and was suspended in 40% polyethylene glycol and 30% ethanol solution. Dantrolene solution (10 mg/ml) was prepared in saline and the solution was subjected to 10-min of sonication just before the injection.

2.3. Acute Isolation of Hippocampal CA1 Neurons and Loading with Fura-2

Acute isolation of CA1 hippocampal neurons was performed by established procedures (Deshpande et al., 2014a; Deshpande et al., 2010; Raza et al., 2004). Briefly, 48-h following POX SE, rats were anesthetized with isoflurane and decapitated. Brains were rapidly dissected and placed in 4 °C oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) consisting of (in mM): 201.5 sucrose, 10 glucose, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 3 KCl, 7 $MgCl_2$, and 0.2 $CaCl_2$). MK-801 (1 μM) was added to all solutions to increase cell viability and was removed 15 min prior to imaging. Hippocampal slices (450 μm) were cut on a vibrating microtome (Leica Microsystems, Wetzlar, Germany) and then equilibrated for 10 min at 34 °C in a piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES)-aCSF solution containing (in mM): 120 NaCl, 25 glucose, 20 PIPES, 5 KCl, 7 $MgCl_2$, and 0.1 $CaCl_2$. Slices were then treated with 8 mg/ml protease in PIPES-aCSF for 6 min at 34 °C and rinsed. Enzyme treated slices were visualized on a dissecting microscope to excise the CA1 hippocampal layer which was then triturated with a series of Pasteur pipettes of decreasing diameter in cold (4 °C) PIPES-aCSF solution containing 1 μM Fura-2 AM (Invitrogen, Carlsbad, CA). The cell suspension was placed in the middle of 2 well glass-bottomed chambers (Nunc, Thermo Scientific). These glass chambers were previously treated overnight with 0.05 mg/ml poly-L-lysine followed by multiple rinses with distilled water and then further treated with Cell-Tak™ (BD-Biosciences, San Jose, CA) biocompatible cellular adhesive (3.5 μg/cm^2) for 30-min, rinsed and air-dried. Neuronal suspension placed in the center of adhesive coated dishes when settled firmly adhered to the bottom. This technique simplified further manipulations on the dissociated neurons. Plates were then incubated at 37 °C in a 5% CO_2/95% air atmosphere for 45 min. Fura-2 was washed off with PIPES-aCSF and plates were incubated an additional 15 min to allow for complete cleavage of the AM moiety from Fura-2. Plates were then incubated at 37 °C in a 5% CO_2/95% air atmosphere for 45 min. Fura-2 was washed off with PIPES-aCSF and plates were incubated an additional 15 min to allow for complete cleavage of the AM moiety from Fura-2.

2.4. Measurement of $[Ca^{2+}]_i$

Following the incubation period, Fura-2 loaded cells were transferred to a 37 °C heated stage (Harvard Apparatus, Hollington, MA) on an Olympus IX-70 inverted microscope coupled to a fluorescence imaging system for $[Ca^{2+}]_i$ measurements (Deshpande et al.,2010; Raza et al., 2004). All experiments were performed using a 20×, 0.7 N.A. water immersion objective and images were recorded by an ORCA-ER high-speed digital CCD camera (Hammamatsu Photonics K.K., Japan). Fura-2 was excited with a 75 W xenon arc lamp (Olympus America, Center Valley, PA). Ratio images were acquired by alternating excitation wavelengths (340/380 nm) by using a Lambda 10-2 filter wheel (Sutter Instruments Co., Novato, CA) and a Fura filter cube at 510/540 emission with a dichroic at 400 nm. All image acquisition and processing was controlled by a computer connected to the camera and filter wheel using Metafluor Software ver 7.6 (MDS Analytical Technologies, Downington, PA). Image pairs were captured every 5 s and the images at each wavelength were averaged over 10 frames. Background fluorescence was obtained by imaging a field lacking Fura-2. Hippocampal CA1 neurons were identified based on their distinct morphology. These neurons displayed pyramidal shaped cell body, long axon and dendrites. The process of enzymatic treatment and mechanical trituration can add minimal stress during acute dissociation of neurons. However, neurons isolated using these procedures exhibit electrophysiological properties identical neurons in slices or in cultures are viable, and not apoptotic or necrotic.

2.5. Calcium calibration

We performed Ca^{2+} calibration determinations as described previously (Deshpande et al., 2010; Raza et al., 2004) to provide estimates of $[Ca^{2+}]_i$ concentrations from the 340/380 ratio values. A Ca^{2+} calibration curve was constructed using solutions of calibrated Ca^{2+} buffers ranging from 0 Ca^{2+} (Ca^{2+} free) to 39 μM Ca^{2+} (Invitrogen, Carlsbad, CA). Values from the calibration curve were used to convert fluorescent ratios to $[Ca^{2+}]_i$. Final $[Ca^{2+}]_i$ were calculated from the background corrected 340/380 ratios using the equation (Grynkiewicz et al., 1985):

$$\left[Ca^{2+}\right]_i = (K_d \times Sf_2/Sb_2) \times (R - R_{min})/(R_{max} - R)$$

where R is the 340/380 ratio at any time; R_{max} is the maximum measured ratio in saturating Ca^{2+} solution (39 μM free Ca^{2+}); R_{min} is the minimal measured ratio Ca^{2+} free solution; Sf_2 is the absolute value of the corrected 380-nm signal at R_{min}; Sb_2 is the absolute value of the corrected 380-nm signal at R_{max}; the K_d value for Fura 2 is 224 nM.