The Role of Intracellular Calcium in Axonal Injury

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***Abstract* — Increased levels of axonal calcium have been found after Traumatic Brain injury. Calcium can enter the cell from an extracellular source or be released from intracellular stores. Here, we investigate the importance of intracellular calcium in axonal pathology by chelating intracellular calcium and isolating components of the injury pathway such as membrane damage, calcium influx, mitochondrial injury, and increased oxidative stress. The findings were that chelating intracellular calcium with BAPTA-AM was neuroprotective after increasing membrane permeability with melittin, damaging mitochondria with CCCP, and increasing oxidative stress with TbHp. Chelating intracellular calcium was not neuroprotective after injuring cells by selectively increasing axonal calcium with A23187.**

I. Background

Brain injury is one of the most common causes of disability and death in adults. Traumatic Brain Injury (TBI) affects approximately 270,000 people a year and costs the U.S. more than $48 billion annually. Around 70,000 people die each year from head injuries, but many of those who manage to survive are left with long-term disabilities as a result of their injuries[1]. While there have been many promising therapeutic treatments for TBI, clinical trials have not been successful. *In vitro* studies can provide insight into the cellular mechanisms leading to neuronal death and degeneration after TBI and aid in developing more effective treatments by isolating components of the injury pathway to investigate the connection between mechanical trauma and resulting pathology.

Collective data has revealed that there are several areas of impairment in TBI, although the connection between them is in many cases unclear. *In vivo* models have demonstrated that after injury there is a loss of integrity in the plasma membrane[2], an increase in intracellular calcium[3], a breakdown of intracellular transport (indicated by focal disruptions in the axonal cytoskeleton and an accumulation of organelles)[4], an increase in oxidative stress[5], mitochondrial damage[6], and the formation of axonal beads[7]

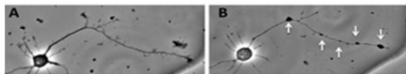

Figure 1. Example of axonal beading in chick forebrain neurons. A) Neuron before injury B) The same neuron 30 minutes after fluid shear stress injury. Beads are denoted by white arrows.

Beading(Figure 1) is a well-documented phenomenon in neurons that correlates with injury severity[7]. It is a term that describes focal bead-like swellings that have been shown in diffuse axonal injury after TBI and other neurological conditions such as epilepsy, Amyotrophic Lateral Sclerosis, and Alzheimer's Disease[7].

Here, we investigate the role of intracellular calcium in several components of a hypothesized TBI pathway. The overall hypothesis is that cell death and dysfunction after TBI are initiated by damage to the plasma membrane. A major consequence of membrane disruption is the loss of ionic homeostasis, resulting in a flux of ions in the direction of their concentration gradient, including an influx of calcium into the cell which can activate various proteases and lead to cell death and dysfunction. The cell attempts to handle the excess intracellular calcium by sequestering it in the mitochondria, which are damaged when overloaded with excess calcium and the increased demand for ATP in order to restore ionic homeostasis. Damage to the mitochondria can result in increased oxidative stress and release of sequestered calcium, propagating the damage cycle.

The approach here is to investigate the role of intracellular calcium by characterizing the response of cells to perturbations of isolated components of the proposed TBI, specifically: membrane damage, increases in intracellular calcium, mitochondrial damage, and oxidative stress and lipid peroxidation. A clear understanding of the injury mechanisms is a requirement for successful therapeutic interventions after injury.

II. Materials and Methods

Glass coverslips were coated overnight with a solution of 0.1 mg/ml of poly-DL-lysine in sodium borate buffer. Embryonic day 8 chick forebrain neurons were dissected, dissociated and plated at a concentration of 1.5 $x10^4$ cells/cm^2[8]. Cultures were maintained in M199 medium and incubated at 37°C and 5% CO_2 for 5 days before experimentation.

Several reagents were chosen to isolate components of the proposed TBI pathway; melittin was used to create pores in the membrane[9], calcium ionophore A23187 was used to increase axonal calcium, carbonyl cyanide 3-chlorophenyl hydrazine (CCCP) was used to damage mitochondria[10], and tert-butyl hydroperoxide (TbHp) was used to increase oxidative stress[11].

Intracellular calcium was chelated using 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis-AM (BAPTA-AM. For all experiments, cells were pre-incubated in BAPTA-AM then incubated with the appropriate chemical insult in combination with BAPTA-AM. The cultures were fixed 0.005% gluteraldehyde for 15 minutes. Cells were imaged using phase microscopy and least 50 cells from 3 coverslips were used for each condition. Beading was used as a measure of injury severity. ImageJ was used to quantify the length and number of beads of axons. Statistical analysis included a Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. All values are presented as means ± SEM.

III. Results and Discussion

Chelating intracellular calcium after increasing membrane permeability with melittin was neuroprotective, reducing the

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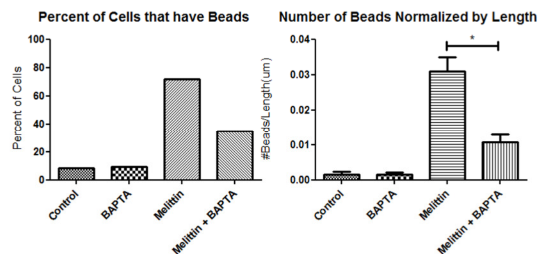

Figure 2. Chelating intracellular calcium is neuroprotective after melittin treatment. Cells were pre-incubated in 100µM BAPTA-AM for 10 minutes, before being treated with 50nM melittin for 30 minutes. ($n \geq 50$, $p < 0.05$)

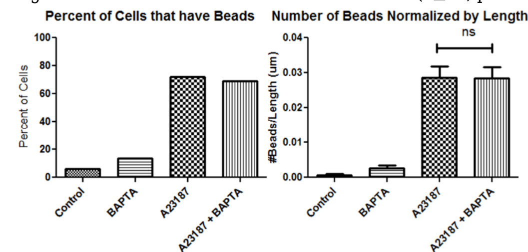

Figure 3. Chelating intracellular calcium is not neuroprotective after A23187 treatment. Cells were pre-incubated in 10 µM BAPTA-AM for 20 minutes before being treated with 10 µM A23187 for 20 minutes. ($n \geq 65$, $p > 0.05$)

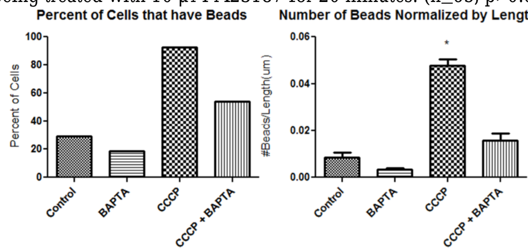

Figure 4. Chelating intracellular calcium is neuroprotective after CCCP treatment. Cells were pre-incubated in 100µM BAPTA-AM before being treated with 100µM CCCP for 60 minutes. ($n \geq 70$, $p < 0.05$)

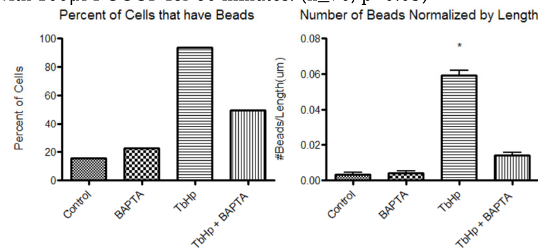

Figure 5. Chelating intracellular calcium is neuroprotective after TbHp treatment. Cells were pre-incubated in 10µM BAPTA-AM for 20 minutes before being treated with 200mM TbHp for 20 minutes. ($n \geq 85$, $p < 0.05$)

percentage of beaded cells and resulting in a statistically significant reduction in beading (Figure 3). In the hypothesized pathway this result would be expected as BAPTA would prevent the propagation of cellular damage by intracellular calcium release. BAPTA was not neuroprotective after increasing axonal calcium using A23187 (Figure 4), which allows the selective entry of calcium.

Chelating intracellular calcium was neuroprotective after damaging mitochondria with CCCP, reducing the percentage of beaded cells and resulting in a statistically significant reduction in beading (Figure 5). This agrees with the hypothesized pathway where sequestered calcium is released as a result of mitochondrial damage as well as with other reports that CCCP causes a release of sequestered mitochondrial calcium [12]. Chelating intracellular calcium was also neuroprotective after increasing oxidative stress using TbHp, reducing the percentage of beaded cells and resulting in a statistically significant reduction in beading (Figure 6).

These results indicate that intracellular calcium plays an important role in axonal pathology after injury. Chelating intracellular calcium reduces beading after membrane damage, mitochondrial damage, and increased oxidative stress. This implies that calcium is released intracellularly during these steps in the pathway.

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References

[1] J. A. Langlois, W. Rutland-Brown, and M. M. Wald, "The epidemiology and impact of traumatic brain injury: a brief overview," *J Head Trauma Rehabil,* vol. 21, pp. 375-8, Sep-Oct 2006.

[2] E. H. Pettus, C. W. Christman, M. L. Giebel, and J. T. Povlishock, "Traumatically induced altered membrane permeability: its relationship to traumatically induced reactive axonal change," *J Neurotrauma,* vol. 11, pp. 507-22, Oct 1994.

[3] Y. Shapira, G. Yadid, S. Cotev, and E. Shohami, "Accumulation of calcium in the brain following head trauma," *Neurol Res,* vol. 11, pp. 169-72, Sep 1989.

[4] E. H. Pettus and J. T. Povlishock, "Characterization of a distinct set of intra-axonal ultrastructural changes associated with traumatically induced alteration in axolemmal permeability," *Brain Res,* vol. 722, pp. 1-11, May 25 1996.

[5] S. L. Smith and E. D. Hall, "Mild pre- and posttraumatic hypothermia attenuates blood-brain barrier damage following controlled cortical impact injury in the rat," *J Neurotrauma,* vol. 13, pp. 1-9, Jan 1996.

[6] K. Dikranian, R. Cohen, C. Mac Donald, Y. Pan, D. Brakefield, P. Bayly, and A. Parsadanian, "Mild traumatic brain injury to the infant mouse causes robust white matter axonal degeneration which precedes apoptotic death of cortical and thalamic neurons," *Exp Neurol,* vol. 211, pp. 551-60, Jun 2008.

[7] H. Takeuchi, T. Mizuno, G. Zhang, J. Wang, J. Kawanokuchi, R. Kuno, and A. Suzumura, "Neuritic beading induced by activated microglia is an early feature of neuronal dysfunction toward neuronal death by inhibition of mitochondrial respiration and axonal transport," *J Biol Chem,* vol. 280, pp. 10444-54, Mar 18 2005.

[8] S. R. Heidemann, M. Reynolds, K. Ngo, and P. Lamoureux, "The culture of chick forebrain neurons," *Methods Cell Biol,* vol. 71, pp. 51-65, 2003.

[9] L. Yang, T. A. Harroun, T. M. Weiss, L. Ding, and H. W. Huang, "Barrel-stave model or toroidal model? A case study on melittin pores," *Biophys J,* vol. 81, pp. 1475-85, Sep 2001.

[10] H. Felle and F. W. Bentrup, "A study of the primary effect of the uncoupler carbonyl cyanide m-chlorophenylhydrazone on membrane potential and conductance in Riccia fluitans," *Biochim Biophys Acta,* vol. 464, pp. 179-87, Jan 4 1977.

[11] Kanupriya, D. Prasad, M. Sai Ram, R. C. Sawhney, G. Ilavazhagan, and P. K. Banerjee, "Mechanism of tert-butylhydroperoxide induced cytotoxicity in U-937 macrophages by alteration of mitochondrial function and generation of ROS," *Toxicol In Vitro,* vol. 21, pp. 846-54, Aug 2007.

[12] D. Murchison and W. H. Griffith, "Mitochondria buffer non-toxic calcium loads and release calcium through the mitochondrial permeability transition pore and sodium/calcium exchanger in rat basal forebrain neurons," *Brain Res,* vol. 854, pp. 139-51, Jan 31 2000.

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