Cortical Degeneration and Contusion Size Is Attenuated in Calpain-1 Knockout Mice Following a Controlled Cortical Impact (CCI)

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INTRODUCTION
An effective pharmacological treatment for individuals suffering from a traumatic brain injury (TBI) has remained elusive. One challenge has been the characterization of numerous proteins that are integral components in the death of neurons following TBI. Calpains, also known as calcium-activated neutral proteases, are involved in various pathological conditions including stroke and TBI (Saatman KE, 1996; Neumar RW, 2003; Saatman KE, 2010).

Currently, 15 mammalian calpains have been identified with calpain-1 and calpain-2 predominantly expressed in the central nervous system (Chong, 2005). Based on in vitro measurements, calpain-1 is activated at micromolar calcium concentrations whereas the calpain-2 activity is detected at millimolar calcium concentrations (Goll DE, 2003). Because of the differential calcium sensitivity, these enzymes are also designated as µ-calpain and m-calpain, respectively. Generally, research has linked calpain-1 and calpain-2 in apoptotic cell signaling, synaptic plasticity, and protein turnover (Altmueller F, 2004; Goll DE, 2003; Wu HY, 2006).

Traditionally, the functional characterization of calpains has been facilitated by the use of synthetic inhibitors and calpastatin, the endogenous protein inhibitor. Calpain inhibition is a possible treatment strategy in reducing the degradation of neuronal and nonneuronal substrates following TBI (Geddes JW, 2010). Post-traumatic inhibition of calpains in animal models of TBI attenuates behavioral deficits, axonal pathology, and cell death (Saatman KE, 2010). However, these inhibitors block the activity of both calpains, leaving the individual role of calpain-1 and calpain-2 poorly understood. Both calpains degrade essentially the same set of substrates in vitro, and intracellular fluctuations of calcium make it difficult to precisely determine the differential activation of each
enzyme in vivo. Therefore, it is essential to develop experimental approaches that can selectively ablate the activity of individual calpains either systemically or in a tissue-specific manner. To address this approach, this study examines the role of calpain-1 in TBI-induced neural degeneration following the controlled cortical impact (CCI) rodent model of TBI, using calpain-1 KO mice.

**MATERIALS & METHODS**

**Animals:** Calpain-1 KO mice were generated using mice with a pure C57BL/6 genetic background by first producing heterozygotes that contained the mutant calpain-1 allele. Through PCR based analysis, mice containing the mutant calpain-1 allele were identified and mated with one another to produce the homozygote calpain-1 KO; Capn1-/-: Calpain-1 KO and C57BL/6 wildtype (WT) mice were housed in the DePaul University Research Facility on a 12:12 light cycle, fed ad libitum and housed 2-4 per cage.

**Controlled Cortical Impact (CCI):** Calpain-1 KO and WT mice (n=6-8) received a CCI unilaterally over the forelimb sensorimotor cortex (FL-SMC). Mice were anesthetized with isofluorane, shaved, and placed in a Kopf stereotaxic apparatus. The skull was exposed and the bite bar adjusted to level bregma and lambda in the horizontal plane. Animals then received a 3 mm diameter craniotomy over the FL-SMC (A/P = +0.3, M/L = +0.15). The impact was delivered by a Benchmark Impactor (Leica), using a 2 mm flat/circular impactor tip at a depth of 0.6 mm below the cortical surface for 250 ms administered at the speed of 3.0 m/s. Contact with the surface of the brain was monitored before decompression using a contact sensor. During the surgery, mice maintained a 37°C body temperature by an automatic heating pad. After the impact, the wound was sutured with monofilament nylon and topical analgesics (Emla cream) and antibiotics were applied. Mice were allowed to recover and were monitored via respiration and righting reflex, before returning to the vivarium.

**Histology:** Mice were sacrificed three days post-CCI by intracardiac perfusion. For histological analysis, the brains were removed and sliced coronally (30 µm) using a cryostat. Sections were then stained with Nissl (using manufacturer’s protocol, Fluoro-Jade C (Histo-Chem; using manufacturers protocol) and TUNEL (Roche; described below).

**Analysis of Contusion Size—**Contusion size was examined by visualizing Nissl stained sections of the FL-SMC using a Leica microscope, a cooled CCD camera, and the software program Neurolucida (Microbrightfield, NJ). Sections containing a contusion cavity between approximately 1.8 mm anterior to bregma and 0.8 mm posterior to bregma were chosen. First, a contour was drawn around the remaining cortex of the injured hemisphere of the brain using a low power objective and the area was obtained. The total area of remaining cortex for all sections was calculated for each animal. A total cortical volume was obtained by multiplying the total cortical area by the distance between successive sections in the set (210 µm).

**Fluoro-Jade C Analysis—**Fluoro-Jade C positive (FJ+) neurons in peri-lesion FLSMC were counted using a Leica microscope, a FITC filter, a cooled CCD camera, and the Neurolucida software program (Microbrightfield, NJ). At a low magnification, the FL-SMC cortex surrounding the contusion was outlined, and the area of the delineated region was determined using the Neurolucida program’s area estimation function. Using a 40X dry objective and the meander scan function in Neurolucida, any FJ+ neurons observed within the outlined area were counted. The number of FJ+ neurons per mm² was calculated as the total number of FJ+ neurons counted divided by the area of the contour in the FL-SMC.

**TUNEL Staining/Analysis—**Tissue sections were stained for apoptosis using a TUNEL stain (Roche, In Situ Cell Death Detection Kit, Fluorescein, version 16.0). Briefly, cryopreserved fixed sections were mounted onto subbed slides, dried, and outlined with a PAP pen.
Slides were then pretreated in 0.3% hydrogen peroxide in methanol for 10 minutes at 15-25°C. Sections were then permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate in phosphate buffered saline (PBS; pH 7.2-7.4) for 20 minutes at 2-8°C, followed by 2 x 5 minutes 0.01M PBS washes. Positive controls were treated with DNase1 (SIGMA AMP-D1; 1U/µl) to produce cleavage in genomic DNA for 1 hour at 37°C in a humidified chamber, followed by 2 x 5 minutes 0.01 M PBS washes. Sections were then labeled with the TUNEL labeling mixture, and incubated in a humidified chamber for 2 hours. One set of DNase treated tissue and injured tissue was designated as negative controls. These tissues were incubated at the same time, but were only treated with the buffer from the labeling mixture. Following labeling, the tissues were rinsed 3x5 minutes in 0.01M PBS. Slides dried for 12 hours in the dark and were cover slipped with Flurosave (Calbiochem). Apoptotic cells were detected using a Leica microscope, FITC Filter, cooled CCD camera and Neurolucida software program (Microbrightfield, NJ). The area surrounding the border of the contusion cavity was outlined using the contour function in Neurolucida using a 10x objective, to obtain a sample area of where TUNEL+ cells were seen. Using a 40x dry objective and the “meander scan function” of Neurolucida, the outlined area was scanned and the number of TUNEL+ neurons in the area was counted. TUNEL+ cells were reported as the total number of TUNEL+ neurons in 12 sections.

Statistics—Contusion size, Fluoro-Jade C, and TUNEL data were analyzed with a One-way ANOVA for group using Microsoft Excel.

RESULTS

Calpain-1 KO mice also exhibited less degenerating neurons compared to WT post-CCI. FJ+ neurons were counted to investigate differences in the number of degenerating neurons in the calpain-1 KO mice. FJ+ neurons were mainly seen in the FL-SMC, surrounding the contusion cavity. Significantly fewer degenerating neurons per mm² were found surrounding the CCI in the calpain-1 KO mice than WT littermates (15.4 ± 5.1 vs. 42.4± 2.7 p<0.002).

Specifically, Calpain-1 KO mice showed significantly less apoptosis compared to WT post-CCI as a form of neuronal degeneration. TUNEL+ neurons were counted in the injured cortex to examine whether calpain-1 played a role in neurodegeneration mediated specifically by apoptosis. TUNEL+ cells were seen primarily within 100µm of the border of the contusion cavity in both WT and calpain-1 KO mice. In a few cases where the contusion cavity reached the corpus callosum, TUNEL+ cells were counted in the corpus callosum as well. The number of TUNEL+ cells in calpain-1 KO mice were significantly decreased as compared to the WT mice (64±21 vs. 167±44 p<0.04).

DISCUSSION

This study demonstrates a functional role of calpain-1 following a rodent model of TBI. Currently, calpain activation has been demonstrated in both dendrites and axons of the cortex and hippocampus in rodent models, post-TBI (Saatman KE, 1996; Zhao X, 1998; Kampfl A, 1996). However, this study is the first to exclusively examine the role of calpain-1 in vivo following TBI. The results show that calpain-1 KO mice demonstrate a significantly decreased contusion size, significantly less neuronal degeneration, and significantly less apoptotic cells at 3 days post-injury in a CCI model. These findings indicate that calpain-1 plays a significant role in the induction of neural degeneration and apoptosis following TBI. Furthermore, this study distinguishes which of the calpain isoforms were specifically implicated in cell death, collectively showing a definitive role for calpain-1 in the induction of apoptosis in vivo following a rodent model of TBI.
Identifying calpain-1 as a major player in the induction of apoptosis makes it an interesting target for inhibition as a potential treatment. A few studies have examined approximately four different calpain inhibitors, with mixed outcomes following animal models of TBI (see review- Saatman KE, 2010). Calpain inhibitor II following a CCI significantly inhibited calpain-mediated spectrin proteolysis in the cortex, suggesting a protective role against cytoskeletal breakdown (Posmantur R, 1997). However, other calpain inhibitors such as AK295 and SJA6017 attenuated behavioral deficits but did not have significant impacts on neuroanatomical measures (Kupina, NC, 2001; Saatman KE, 2000). The contradicting effects of these studies may result from the inhibitors non-specificity to individual calpain isoforms. The structural and functional diversity of calpains in cells is reflected in their involvement in the pathogenesis of a wide range of disorders in a tissue-specific manner. The data obtained shows that TBI-induced pathology is linked to calpain-1 activation. Suggesting that pharmacological inhibition of calpain-1 activity could provide a therapeutic benefit for TBI and other head traumas. However, current pharmacological calpain inhibitors are not specific enough to inhibit individual isoforms. Therefore, until technical advances in procedures have improved calpain-1 inhibitors, an alternative approach for therapeutic benefit could reside in inhibiting the translocation of calpain-1 into mitochondria—an essential step for cleaving AIF. Additionally, improvements of gene therapy or siRNA approaches to specifically knock down or silence calpain-1 may also be a viable option in models of TBI and neurodegenerative diseases. Future studies will examine the signaling processes by which calpain-1 mediates neuronal injury and its potential role as a biomarker/therapeutic target.

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FIGURE 1
Calpain-1 KO mice exhibit a significantly larger volume of remaining cortex compared to WT littermates (E; *p<0.02). This parameter indicates a smaller contusion. The smaller contusion in calpain-1 KO mice is demonstrated by surface area pictures of the contusion prior to sectioning (A&B), and in Nissl stained coronal sections through the center of the contusion- 2.5Xmag (C&D).
The total number of TUNEL+ neurons surrounding the border of the contusion is significantly decreased in calpain-1 KO mice compared to WT (D; *p<0.04).

**FIGURE 2**

FJ+ neurons are observed surrounding the contusion site in both WT (A) and calpain-1 KO (B) mice. However, the density of FJ+ neurons is significantly reduced in calpain-1 KO mice (C; *p<0.002). Scale = 25 μm.

**FIGURE 3**

The total number of TUNEL+ neurons surrounding the border of the contusion is significantly decreased in calpain-1 KO mice compared to WT (D; *p<0.04).

**FIGURE 4**

Animals received a CCI over their FL-SMC (B), delivered by a Benchmark Impactor (A).
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