Targeted Gene Inactivation of Calpain-1 Suppresses Cortical Degeneration Due to Traumatic Brain Injury and Neuronal Apoptosis Induced by Oxidative Stress*

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Background: Calpains play an important role in the regulation of cell death. Results: Calpain-1 inhibition decreases cortical neurodegeneration following TBI by regulating calcium influx and apoptosis of neurons under oxidative stress. Conclusion: Genetic inhibition of calpain-1 reduces neurodegeneration and suppresses neuronal apoptosis. Significance: Targeted inhibition of calpain-1 offers a promising therapeutic approach against TBI and other neurodegenerative diseases.

Calpains are calcium-regulated cysteine proteases that have been implicated in the regulation of cell death pathways. Here, we used our calpain-1 null mouse model to evaluate the function of calpain-1 in neural degeneration following a rodent model of traumatic brain injury. In vivo, calpain-1 null mice show significantly less neural degeneration and apoptosis and a smaller contusion 3 days post-injury than wild type littermates. Protection from traumatic brain injury corroborated with the resistance of calpain-1 neurons to apoptosis induced by oxidative stress. Biochemical analysis revealed that caspase-3 activation, extracellular calcium entry, mitochondrial membrane permeability, and release of apoptosis-inducing factor from mitochondria are partially blocked in the calpain-1 null neurons. These findings suggest that the calpain-1 knock-out mice may serve as a useful model system for neuronal protection and apoptosis in traumatic brain injury and other neurodegenerative disorders in which oxidative stress plays a role.

Apoptosis is observed in various neurological and neurodegenerative disorders, such as Alzheimer, Huntington, and Parkinson diseases, amyotrophic lateral sclerosis, stroke, ischemia, spinal cord injury, and traumatic brain injury (TBI) (1). The signals initiating neuronal apoptosis include the following: (a) lack of neurotrophic factor support; (b) overactivation of excitatory neurotransmitter glutamate receptors; and (c) an increased oxidative stress. In fact, the brain is highly sensitive to oxidative stress because it has the highest metabolic rate in any organs of the body, an increased amount of unsaturated fatty acids, and high levels of iron (2). However, this sensitivity may result from an inadequate defense system against oxidative stress because the brain has significantly lower catalase activity than other organs (2). Therefore, it is of much interest to investigate the molecular pathways of apoptosis in neurons subjected to oxidative stress. This analysis may unveil new molecular approaches for the treatment of neurodegenerative diseases and neural injury.

Increased oxidative stress followed by calcium influx, mitochondrial dysfunction, and the loss of cytoskeletal proteins are phenotypes commonly observed in Alzheimer, Huntington, and Parkinson diseases, as well as amyotrophic lateral sclerosis, stroke, ischemia, spinal cord injury, and TBI (1, 3, 4). An elevated intracellular calcium concentration will hyper-activate calcium-activated neutral proteases called calpains. Activation of calpains is involved in various pathological conditions, including ischemic and traumatic brain injuries (5–7). Importantly, calpain inhibitors are neuroprotective in the free radical injury models associated with mitochondrial dysfunction (8), apoptotic injury following spinal cord trauma (9), and ischemic and TBI animal models (10, 11).

Calpains have been shown to play a role in many different models of TBI. In models of diffuse and focal TBI, calpain-mediated spectrin breakdown products as well as expression of m- and μ-calpain are shown to increase significantly in the cortex and hippocampus within the first 24 h post-injury (5, 12, 13). The addition of calpain inhibitor II following a focal, controlled cortical impact (CCI) model of TBI significantly inhibited calpain-mediated spectrin proteolysis in the cortex sug-
suggesting a protective role against cytoskeletal breakdown (14). However, other calpain inhibitors (AK295 and SJA6017) attenuated behavioral deficits but did not have significant impact on neuroanatomical measures (15, 16). Similarly, previous studies have examined at least four different calpain inhibitors using TBI animal models with mixed outcomes (7). The differential outcome of these studies is most likely due to the nonspecificity of inhibitors for a calpain isoform and inhibition of enzymes, including cathepsins. In this study, we sought to elucidate a specific role of calpain-1 in cortical neurodegeneration following the CCI model of TBI using the calpain-1 knock-out (KO) mouse model. The CCI model of TBI results in a reproducible area of cell death that forms a measurable cavity in the cortex (17, 18) within the first 24–48 h post-impact. The main mechanism by which cell death occurs in CCI has been demonstrated in numerous studies to be apoptosis (19–21), and one potential mechanism by which apoptosis occurs post-TBI is via oxidative stress (22). This, together with the previous examination of various calpain inhibitors in this model, makes it an ideal choice for further elucidation of the roles of individual calpains in neuronal death pathways.

Despite extensive studies linking the activation of calpains to neurodegenerative diseases, relatively little is known about the molecular mechanisms of calpain-mediated apoptosis of neurons under physiological conditions. To date, many calpain substrates have been identified, and some of them are known to play key roles in cell death pathways (10). These substrates have been identified, and some of them are known to play key roles in cell death pathways (10). These substrates include caspases (23, 24), Bax (25, 26), Bid (27, 28), and AIF (29, 30). However, the precise mechanism linking individual calpain isoforms to oxidative stress and apoptosis pathways remains unclear.

In this study, we investigated the function of calpain-1 in neurodegeneration induced by TBI and neuronal apoptosis induced by oxidative stress. Our results show that calpain-1 significantly decreases cortical apoptosis and neurodegeneration in vivo following TBI. Our in vitro studies demonstrate the potential molecular mechanism of calpain-1 in apoptosis and show that it plays a functional role in the regulation of extracellular calcium influx and apoptosis in primary neurons exposed to oxidative stress. Together, these findings reveal a physiological role of calpain-1 in the regulation of calcium influx and apoptosis of primary neurons subjected to oxidative stress and in neuronal death following traumatic brain injury.

**EXPERIMENTAL PROCEDURES**

**Animals**

The calpain-1 null mouse model (KO) was originally developed in our laboratory by traditional genetic inactivation methods using homologous recombination in embryonic stem cells (31). Briefly, a targeting vector was engineered that deleted the 5’ segment of exon 4 (amino acids 153–160 encoding LWQF-GEWV) and disrupted the gene by inserting a pGK-Neo cassette. Exon 4 encodes a critical part of the catalytic domain of calpain-1. Northern blot analysis indicated a complete absence of the calpain-1 transcript in both erythroblast and nonerythroblast tissues. Absence of the calpain-1 transcript was further confirmed by RT-PCR analysis of liver, lung, and kidney tissues. Further analysis indicated that the transcripts of calpain-2 and the regulatory 30-kDa subunit of calpains were unaltered in the calpain-1 null tissues (31). We determined the brain weights of intact mice in both genotypes. There was no significant difference between them. The calpain-1 KO mice used in this study were back-crossed at least 20 generations using the wild type C57BL/6 mice (32). The calpain-1 KO mice, now on nearly pure C57BL/6 genetic background, were used in this study. Calpain-1 KO and C57BL/6 wild type (WT) mice were transported from the University of Illinois to DePaul University and quarantined for a week in the DePaul University Research Facility prior to housing. Animals were kept on a 12:12 light cycle, fed *ad libitum*, and housed 2–4 mice per cage. All experiments were conducted using both National Institutes of Health and Institutional guidelines for the care and use of animals in research.

**In Vivo Study Methods**

**CCI**—Calpain-1 KO and WT mice (n = 6–8) received a CCI unilaterally over the forelimb sensorimotor cortex (FL-SMC) (33). Mice were anesthetized with isoflurane and placed in a Kopf stereotaxic apparatus and shaved. 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 speed of 3.0 m/s and a depth of 0.6 mm below the cortical surface for 250 ms. Contact with the brain was monitored by a contact sensor before decompression was administered. After the impact, the wound was sutured with monofilament nylon followed by application of topical analgesics (Emla cream) and antibiotics. Mice were allowed to recover and were monitored by respiration and righting reflex, before returning to the vivarium. During the surgery, mice maintained a 37 °C body temperature by an automatic heating pad.

**Sacrifice**—Animals were sacrificed 3 days post-CCI by intracardiac perfusion with PBS + 0.01% heparin (in PBS) followed by 4% buffered paraformaldehyde solution. The time point chosen in this study was 3 days post-injury to examine contusion size, neurodegeneration, and apoptosis at its peak (17–21).

**Histology**—Brains were removed and sliced coronally (30 μm) into six sections of sections using a cryostat. One set of sections was stained with Nissl (using manufacturer’s protocol), one with Fluoro-Jade® C (Histo-Chem Inc.; using manufacturer’s protocol), and one with TUNEL (Roche Applied Science; 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 ~1.8 mm anterior to bregma and 0.8 mm posterior to bregma were chosen. After calibrating the software, 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 FL-SMC were counted using a Leica microscope, a FITC filter, a cooled CCD camera, and the NeuroLucida software program (Microbrightfield, NJ). At a lower magnification, the FL-SMC cortex surrounding the contusion was outlined, and the area of the delineated region was determined using the NeuroLucida program area estimation function. Using a ×40 dry objective and the meander scan function in NeuroLucida, any FJ+ neurons observed within the previously outlined area were counted. The number of FJ+ neurons/mm² was calculated as the total number of FJ+ neurons counted divided by the area of the contour in the FL-SMC.

**TUNEL Staining**

Tissue sections from injured mice were stained for apoptosis using a TUNEL stain (In Situ Cell Death detection kit, fluorescein, Version 16.0, Roche Applied Science). Briefly, cryopreserved fixed sections that were within the anterior-posterior boundaries of the contusion as delineated in the contour size analysis (12 sections) were mounted onto subbed slides, allowed to dry, and outlined with a PAP pen. The slides were then pretreated in 0.3% hydrogen peroxide in methanol for 10 min 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 min at 2–8 °C, followed by two washes for 5 min in 0.01 M PBS. Sections designated as positive controls were treated with DNase I (Sigma AMP-D1, 1 unit/μl) to produce cleavage in genomic DNA for 1 h at 37 °C in a humidified chamber, followed by 2 washes for 5 min in 0.01 M PBS washes. Sections were then labeled with the TUNEL labeling mixture and incubated in a humidified chamber for 2 h. One set of DNase-treated tissue and one set of injured tissue were also designated as negative controls. These tissues were incubated at the same time but only in the buffer used in the labeling mixture. Following labeling, the tissues were rinsed three times for 5 min in 0.01 M PBS. Slides dried for 12 h in the dark and were then coverslipped with FluorSave (Calbiochem). Apoptotic cells were detected using a Leica microscope, FITC filter, cooled CCD camera, and NeuroLucida software program (Microbrightfield, NJ). Because most of the TUNEL+ cells were seen surrounding the border of the contusion cavity, an area surrounding the border of the contusion was outlined using the contour function in NeuroLucida using a ×10 objective. Using a ×40 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. The data are reported as the total number of TUNEL+ neurons sampled in 12 sections.

**Statistics**

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

**In Vitro Study Methods**

**Antibodies and Reagents**—Rabbit polyclonal antibodies against calpain-1 (Calbiochem), calpain-2 (Sigma), AIF (Cell Signaling), mouse monoclonal voltage-dependent anion-selective channel protein 1 (Abcam), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology), goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody, and anti-mouse HRP conjugated antibody (Upstate) were obtained commercially.

**Culture of Primary Cortical Neurons**—Calpain-1 heterozygous (+/-) females were mated with calpain-1 heterozygous males to generate WT, heterozygous, and KO mice within the same litter. Primary cortical neurons were cultured as described previously (34). The cortical neurons isolated from each embryo were plated at a density of 1,500 cells/mm² in the plating medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) and 5% horse serum) and mounted onto glass coverslips coated with poly-L-lysine (Sigma). After the attachment of neurons to the substrate, the medium was exchanged to neuronal culture medium (minimal essential medium (MEM) with B27 supplement, 0.6% glucose, 1.0 mM sodium pyruvate, and 2 mM GlutaMAX). One-third of the medium was changed with fresh medium every 2 days. Genomic typing was performed by PCR (32).

**Induction of Oxidative Stress**—To induce oxidative stress-mediated neuronal injury, cultured neurons were exposed to 100 μM H₂O₂ for 5 min in MEM at 7–11 days of culture in vitro (DIV). Control cultures were treated with MEM alone. The 5-min exposure of neurons to oxidative stress is referred to here as the “induction of apoptosis.” After the exposure, cells were returned to the original culture medium and incubated for 6–24 h.

**TUNEL Assay**—24 h after the induction of apoptosis, cells undergoing DNA fragmentation were detected using the TUNEL kit (Roche Applied Science) according to the manufacturer’s protocol.

**Flow Cytometric Analysis of Cleaved Caspase-3 and Mitochondrial Membrane Potential**—Cells were treated with either 100 μM calpain inhibitor MDL28170 (MDL, Aventis Pharmaceuticals) or 1.0 μM cyclosporin A (CsA, Sigma) for 1 h prior to the induction of apoptosis. Cells were exposed to 100 μM H₂O₂ for 5 min, returned to the original culture medium, and incubated for an additional 6 h. Cells were stained with phycoerythrin (PE)-conjugated antibody against active caspase-3 according to the manufacturer’s protocol (BD Biosciences). Cells were then analyzed by flow cytometry using an LSR (BD Biosciences). Mitochondrial membrane potential was measured by Rhodamine 123 (Rh123, Sigma) with an LSR (BD Biosciences). Cells suspended in PBS were supplemented with 2% FCS and incubated with 10 μM of Rh123 at 37 °C for 10 min. Cells were washed with ice-cold PBS and analyzed by flow cytometry.

**Mitochondrial Fractionation**—6 h after the induction of apoptosis, cells were separated into mitochondria, nuclei, and cytosol fractions using a fractionation kit (Qiagen).

**Measurement of Caspase-3 Activity**—Six hours after the induction of apoptosis, the enzyme activity of caspase-3 was measured in the cell lysate using a caspase-3 colorimetric assay kit (R&D Systems; BF3100). Protein concentration in the cell lysate was measured by the BCA protein assay kit (Pierce). The enzyme reaction for caspase activity was carried out in a 96-well
Knockdown by siRNA—Primary cortical neurons were plated with a density of $3 \times 10^5$ cells/well on coverslips coated with 0.1 mg/ml poly-L-lysine in a 24-well plate. The next day, the medium was changed to neuronal growth medium. The cells were then transfected with 10 pmol of siRNA (sc-29886 and sc-41460, Santa Cruz Biotechnology) and 1.0 μl of Lipofectamine (Invitrogen) in 50 μl of OptiMEM. Cells were exposed to 200 μM H$_2$O$_2$ for 10 min at DIV7 in MEM. Cells were returned to the culture medium after H$_2$O$_2$ exposure, and incubated for 24 h. Apoptotic cell ratio was measured by the TUNEL assay kit (Roche Applied Science). The knockdown of calpain-1 and calpain-2 was analyzed by Western blots using the respective antibodies. A nonsilencing siRNA obtained from Qiagen was used as a negative control.

Cytosolic Ca$^{2+}$ Measurements—H$_2$O$_2$-induced increase in the cytosolic Ca$^{2+}$ concentration [Ca$^{2+}$]$_i$ was measured using the Ca$^{2+}$-sensitive fluorescent dye Fura-2 acetoxyethyl ester (35). Primary cortical neurons from WT and calpain-1 KO embryos were cultured in a glass bottom dish coated with 0.1 mg/ml poly-L-lysine. At DIV7, cells were incubated in the medium without B27 supplement for 30 min. Cells were then washed twice with Hanks’ buffered salt solution and loaded with 3.0 μM Fura-2 acetoxyethyl ester for 30 min. After the loading, cells were washed with Hanks’ buffered salt solution, and the coverslips were transferred to a perfusion chamber and imaged using an Axio observer D1 semi-motorized microscope (Carl Zeiss, Sartrouville, France) equipped with a camera and a Fluor 40× oil immersion objective lens (Carl Zeiss). The light beam was controlled by a DG-4 wavelength switcher (Princeton Instruments). A dual excitation mode at 340 and 380 nm was used, and the emission signal was collected at 520 nm. The software AxioVision Physiology Module was used to acquire the images at a frequency of 1.0-s intervals, and the data were analyzed off line. Upon excitation at 340 and 380 nm, the regions of interest in individual cells were marked, and the emission signal was collected at 520 nm with a 5-s interval. In each experiment, 20–30 cells were selected and analyzed to measure the change in [Ca$^{2+}$]$_i$.

RESULTS

In Vivo Study

Calpain-1 KO Mice Show Significantly Smaller Contusions Than WT Littermates—The volume of remaining cortex was examined to determine contusion size in both WT and calpain-1 KO mice. A larger volume of remaining cortex corresponds to a smaller contusion. As shown in Fig. 1, calpain-1 KO showed an increase in the volume of remaining cortex, i.e. a smaller contusion as compared with WT mice (14.7 ± 0.19 mm$^3$ versus 13.1 ± 0.16 mm$^3$, $p < 0.02$).

Calpain-1 KO Mice Exhibit Less Degenerating Neurons Compared with 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/mm$^2$ were found surrounding the CCI in the calpain-1 KO mice than WT littermates (15.4 ± 5.1 versus 42.4 ± 2.7; $p < 0.002$) (Fig. 2).

Calpain-1 KO Mice Show Significantly Less Apoptosis Compared with WT Post-CCI—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 100 μm 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 also present in the corpus callosum. However, the number of TUNEL$^+$ cells in the calpain-1 KO mice were significantly decreased as compared with the WT mice (64 ± 21 versus 167 ± 44; $p < 0.04$; Fig. 2).

In Vitro Study

The results from the in vivo study indicate that calpain-1 plays a significant role in apoptosis-mediated neuronal death following TBI. To better elucidate the mechanisms by which calpain-1 mediates apoptosis, cells from these mice were further examined in a series of in vitro experiments.

DNA Fragmentation Induced by Oxidative Stress Is Significantly Decreased in Calpain-1 KO Neurons—To investigate the role of calpain-1 in neuronal apoptosis, we isolated cortical neurons from calpain-1 KO embryos and WT littermates. Neurons were exposed to H$_2$O$_2$ for 5 min, followed by incubation in the culture medium for 24 h. The DNA fragmentation, a hallmark of apoptosis, was analyzed by the TUNEL assay (Fig. 3, A and B). WT neurons showed extensive DNA fragmentation (41.1 ± 4.2%) as compared with neurons from calpain-1 KO mice (17.5 ± 5.0%) (Fig. 3, A and B). More than 500 TUNEL-positive cells were counted for each measurement, and experiments were repeated at least four times. It is to be noted that the protein expression level of calpain-1 in the heterozygous neurons is comparable with WT neurons (Fig. 3C). Therefore, the apoptosis phenotype of heterozygous neurons was similar to WT cells under these conditions of oxidative stress (data not shown). The protein expression of calpain-2 and caspase-3 was unchanged in the calpain-1 KO neurons (Fig. 3C), indicating that the calpain-2 and caspase-3 do not compensate for the absence of calpain-1. Similarly, the cleavage of caspase-3 under basal conditions was unchanged (Fig. 3C), consistent with the measurements by flow cytometry (Fig. 4B). Thus, the calpain-1 KO neurons show resistance to apoptosis induced by oxidative stress, suggesting a functional role of calpain-1 in the regulation of neuronal apoptosis pathway.

Calpain-1 Induces Caspase-3 Activation—To investigate the upstream components of the apoptosis pathway, we examined the activation of caspase-3 in calpain-1 KO neurons as well as WT and heterozygous littermates. Neurons were exposed to oxidative stress for 5 min, incubated for 6 h in the culture medium without oxidative stress, and then stained with an antibody specific for cleaved caspase-3. The antibody was conjugated with PE. Flow cytometry revealed that under basal conditions, the WT, heterozygous, and calpain-1 KO neurons show no significant difference in the level of cleaved caspase-3 (Fig. 4, A and B). Interestingly, the number of cleaved caspase-3-positive cells increased with oxidative stress in the WT and heterozygous neurons, whereas the calpain-1 KO neurons...
exhibit significantly less cleavage of caspase-3 (Fig. 4B). These results suggest a functional role of calpain-1 in the activation of the caspase cascade. Furthermore, to investigate the role of calpain-2 in this pathway, we treated the WT, heterozygous, and calpain-1 KO neurons with a pan-calpain inhibitor, MDL, which inhibits both calpain-1 and calpain-2. The cleavage of caspase-3 after H2O2 stimulation in the WT and heterozygous neurons was significantly inhibited by the MDL, whereas its effect was minimal in the calpain-1 KO neurons (Fig. 4B). The minimal inhibitory effect of MDL observed with the calpain-1 KO neurons could be mediated by calpain-2 present in the calpain-1 KO neurons.

The cleavage of caspase-3 is considered to be the initial step for its activation. However, when caspase-3 is bound to its physiological inhibitor, XIAP, it does not show the protease activity even upon its cleavage. To overcome this limitation, we measured the enzyme activity of caspase-3 in the WT and calpain-1 KO neurons using the caspase-3-specific peptide, DEVD, conjugated to a color reporter p-nitroaniline. The H2O2 treatment of cells induced caspase-3 activation in the WT neurons, whereas the activation of caspase-3 was significantly reduced in the calpain-1 KO neurons (Fig. 4C). These results suggest that caspase-3 is activated via the calpain-1-dependent pathway under the specified conditions of oxidative stress.

**FIGURE 1.** Volume of remaining cortex is significantly larger in the calpain-1 KO mice compared with WT littermates (E; *, p < 0.02). This parameter indicates a smaller contusion. The smaller contusion in calpain-1 KO mice is demonstrated by surface pictures of the contusion prior to sectioning (A and B) and in Nissl-stained coronal sections through the center of the contusion (C and D). The sample sizes are KO = 7 and WT = 6.
Calpain-1 Regulates Calcium Entry—To investigate if the H$_2$O$_2$ treatment regulates calcium influx in the WT and calpain-1 KO neurons, the cytosolic Ca$^{2+}$ concentration [Ca$^{2+}$]$_i$ was measured in the presence and absence of H$_2$O$_2$. In the presence of H$_2$O$_2$, the addition of 1.5 mM calcium to Fura-2-loaded WT neurons resulted in a large rise in [Ca$^{2+}$]$_i$ (Fig. 5A). In contrast, the rise in [Ca$^{2+}$]$_i$ was significantly reduced in the calpain-1 KO neurons (Fig. 5A). This observation suggests that either calpain-1 KO neurons have a defect in the H$_2$O$_2$-induced calcium entry pathway or they undergo a relatively faster removal of excess calcium from the cytosol. To further evaluate if the lack of calpain-1 in neurons affects their basal calcium concentration, the cells were first bathed in the calcium-free buffer and then incubated with a solution of 1.5 mM calcium. Interestingly, the basal calcium entry into the cytosol was not altered in the WT and calpain-1 KO neurons (Fig. 5B). This finding suggests that the calpain-1 KO cells most likely show a defect in the H$_2$O$_2$-induced Ca$^{2+}$ entry pathway, thus indicating a novel role of calpain-1 in the regulation of calcium entry in neurons.

Calpain-1 Mediates Mitochondrial Membrane Permeability—The upstream regulators of caspase-3 activation include caspase-9, which is activated by the release of cytochrome c, Apaf-1, and the dATP complex. Because the release of cytochrome c from mitochondria is induced by the loss of mitochondrial membrane potential (MMP), we measured the MMP by flow cytometry using Rh123 in neurons after oxidative stress (Fig. 6). The uptake of lipophilic cation, Rh123, by mitochondria is proportional to mitochondrial membrane potential (36). The number of Rh123-positive cells was significantly reduced in the heterozygous neurons, suggesting a loss of MMP by oxidative stress (Fig. 6). Interestingly, the calpain-1 KO neurons retained the same level of Rh123 signal in the mitochondria as the heterozygous neurons treated with the pan-calpain inhibitor MDL (Fig. 6). Moreover, we used CsA as an internal positive control for the heterozygous neurons subjected to oxidative stress (Fig. 6). The CsA functions as an inhibitor by preventing the opening of the mitochondrial permeability transition pore (37). Because the MDL did not affect MMP in the calpain-1 KO neurons, this observation suggests that the oxidative stress...
induces mitochondrial permeability in WT neurons via the calpain-1 but not calpain-2 pathway.

**Calpain-1 Regulates AIF Release from Mitochondria**—Besides caspase-3, AIF is also known to mediate apoptosis and DNA fragmentation in many cell types. Therefore, we examined AIF release in the calpain-1 KO and heterozygous neurons (Fig. 7, A and B). Upon oxidative stress, the AIF release from mitochondria was observed in the heterozygous/KO/WT neurons. The released form of AIF is slightly smaller than the size of AIF in mitochondria, consistent with the evidence that AIF is released from mitochondria after truncation by calpains (38). Interestingly, the release of AIF from mitochondria into cytosol was significantly reduced in the calpain-1 KO neurons (Fig. 7B). However, the truncation and release of AIF were not completely abolished in the calpain-1 KO neurons presumably due to the presence of calpain-2 and cathepsins. These findings suggest that the truncation and release of AIF from mitochondria is partially blocked by the inactivation of calpain-1 in mouse neurons. Based on these observations, we conclude that the calpain-1 plays an essential role in the regulation of neuronal apoptosis mediated by oxidative stress under the specified experimental conditions.

**DISCUSSION**

This report is the first to demonstrate a functional role of calpain-1 following an animal model of TBI. Although other studies have examined the general role of calpains following TBI (7), none have exclusively examined the role of calpain-1 in vivo. Our study has shown that in the CCI model of TBI, calpain-1 knock-out mice demonstrate a significantly decreased contusion size, significantly less neuronal degeneration, and significantly less apoptotic cells at 3 days post-injury (Figs. 1 and 2). The effect at this acute time point is important because it typically represents the peak of cell death in this injury model (19). These findings indicate that calpain-1 plays a significant role in the induction of neural degeneration and apoptosis following TBI. Although previous studies have demonstrated that calpains in general are increased at early time points following TBI. Although previous studies have demonstrated that calpains in general are increased at early time points following this model of TBI (5, 12, 13), they were not able to distinguish which of the calpain isoforms were specifically implicated in cell death. This study shows a definitive role for calpain-1 in the induction of apoptosis in vivo following a focal rodent model of TBI.

Calpains are known to be involved in the regulation of apoptosis of many cell types, including neurons in vitro, under various stress stimuli such as oxidative stress, inflammatory stress, and others (5, 9, 39, 40). Here, we provide biochemical evidence for a functional role of calpain-1 in neuronal apoptosis mediated by oxidative stress that may underpin the mechanistic basis of neurodegeneration following TBI. Along with the lack of neurotrophic support, excessive activation of excitatory neurotransmitter receptors, and a heightened inflammatory response, the exposure of brain to oxidative stress is thought to play a significant role in cell death following TBI (22). Our study has shown that the calpain-1 KO neurons exposed to oxidative stress...
stress are resistant to apoptosis as measured by TUNEL, show reduced calcium entry, exhibit decreased mitochondrial membrane permeability, suppress the release of AIF from the mitochondria, and significantly decrease the cleavage of caspase-3. These findings correlate with a significant reduction of the neurodegenerative and apoptotic response of calpain-1 KO mice to TBI, as evident in the current study. Specifically, these mechanisms have been shown to play a role in TBI-induced degener-

**FIGURE 4. Flow cytometric analysis of apoptotic and nonapoptotic populations using anti-active caspase-3 antibody.** A, detection of cleaved caspase-3-positive cells in cultured neurons by flow cytometry. Six hours after oxidative stress, neurons were analyzed using an anti-cleaved caspase-3 antibody conjugated with PE. MDL is a pan-calpain inhibitor. DMSO is used as a vehicle. The histogram shows the intensity of PE (x axis; 10^0, 10^1, 10^2, and 10^3 from the left) and the number of events detecting the cells with the intensity plotted on the y axis. Right peak represents the cleaved caspase-3-positive cells. B, ratio of cleaved caspase-3-positive cells is plotted versus the total number of cells. PE-positive cells were represented as means ± S.E. (WT, 25%; WT + H_2O_2, 36 ± 2.7%; WT + H_2O_2 + MDL, 29 ± 1.2%; KO, 25 ± 0.21%; KO + H_2O_2, 27 ± 2.8%; KO + H_2O_2 + MDL, 22%). The p value was calculated by one-way ANOVA using Tukey-Kramer multiple comparisons test with Graphpad InStat. Asterisks indicate the pair of samples having a p value of less than 0.05. The experiments were repeated at least twice. C, activity of caspase-3 measured by the caspase-3-specific peptide (DEVD) conjugated with the reporter molecule p-nitroaniline. The cleavage of the peptide by the caspase-3 was quantified spectrophotometrically at a wavelength of 405 nm. Relative activity of caspase-3 compared with untreated WT was shown in the graph (WT + H_2O_2, 2.4 ± 0.02; KO, 0.57 ± 0.01; KO + H_2O_2, 1.32 ± 0.06). The experiments were repeated at least twice. Two-way ANOVA was performed for significance. F values of treatment, genotype, and their interaction were 3.617, 1.784, and 331, respectively, which were greater than the F critical 7.7. Post hoc multiple comparisons were performed using the Bonferroni method with GraphPad InStat. Asterisks indicate the pair of samples having a p value of less than 0.05.

**FIGURE 5. Calpain-1 regulates Ca^{2+} entry.** A, at DIV7, neurons were loaded with Fura-2 acetoxymethyl ester and used to measure the H_2O_2-induced increase in [Ca^{2+}]. Solid and dotted lines represent the 340:380 ratio of WT and calpain-1 KO, respectively. More than 40 neurons were analyzed, and average values with standard error are shown as a graph. B, similarly, basal Ca^{2+} entry without H_2O_2 was measured in the WT and calpain-1 KO neurons.
Calpain-1 Regulates Degeneration and Apoptosis Post-TBI

Calpain-1 mediates the loss of mitochondrial membrane potential. Six hours after oxidative stress, neurons were incubated with Rh123. After washing, the remaining Rh123 signal in mitochondria reflecting the mitochondrial membrane potential was analyzed by flow cytometry. Cyclosporin A is an inhibitor for mitochondrial membrane permeabilization. MDL is an inhibitor for calpains. Quantitative analysis of the relative ratio of Rh123-positive cells is shown in the graph as means ± S.E. (heterozygous 1; heterozygous + H2O2; 0.63 ± 0.01; heterozygous + H2O2 + ML, 0.95 ± 0.10; KO, 0.91 ± 0.09; KO + H2O2, 0.89 ± 0.01; KO + H2O2 + ML, 0.82 ± 0.06). The experiments were repeated at least twice. The p value was calculated by Tukey-Kramer one-way ANOVA using Graphpad InStat. Asterisks indicate the pair of samples having a p value of less than 0.05.

It has been suggested that oxidative stress induces calcium influx in several neurodegenerative diseases causing activation of calpains and other enzymes (1, 3). Our findings indicate that calpain-1 KO neurons show a defect in the calcium entry pathway mediated by oxidative stress (Fig. 5). Calpains are known to cleave over 40 substrates in vivo. However, it is unclear which of these substrates can mediate the functional involvement of calpain-1 in calcium entry into neurons. Previous studies have suggested a role of calpains in the store-operated Ca2+ entry mechanisms (45). It was suggested that the calpains may modulate store-operated Ca2+ entry mechanisms through the regulation of a protein tyrosine phosphorylation cascade and the reorganization of the cortical actin cytoskeleton in platelets (46). A similar role for calpains in cytoskeletal reorganization was demonstrated in neurons (47). Several calpain substrates have been implicated in the regulation of cell adhesion and motility processes (10, 48). Our findings are consistent with the idea that calpain-1 lies upstream of the protein tyrosine phosphorylation cascade required for the activation of calcium entry (46). It is plausible that a lack of remodeling of the cortical cytoskeleton may uncouple inositol triphosphate receptor and transient receptor potential channel 1 (TRPC1), a Ca2+ channel, in the calpain-1 KO neurons (46). This uncoupling would be a prerequisite for calcium entry mechanisms (35).

Besides calcium channels, the Na+/Ca2+ exchanger also plays an important role in the regulation of calcium levels (49).
Previously, it was shown that calpains cleave the Na+/Ca2+ exchanger; and this cleavage elevates the level of calcium by inhibiting the removal of intracellular calcium (50). Therefore, there remains a possibility that the cleavage of the Na+/Ca2+ exchanger might be impaired in the calpain-1 KO neurons. The reduced cleavage of the Na+/Ca2+ exchanger may potentiate the removal of cytosolic calcium from calpain-1 KO neurons, thus masking the postulated defect in the calcium entry pathway. Based on the published evidence and our findings, we propose that calpain-1 activation mediates calcium entry under oxidative stress, further amplifying the calpain-1 activity, and thus forming a positive feedback loop. Future studies using the calpain-1 KO mouse model will clarify these predictions.

Oxidative stress induces mitochondrial membrane permeabilization causing peroxidation of membrane lipids, inhibiting enzymes in the electron transport chain of the mitochondria, resulting in a blockage of mitochondrial respiration (2). The calpain-1 KO neurons show a defect in mitochondrial membrane permeability under oxidative stress (Fig. 6), suggesting that the permeability is at least partially regulated by calpain-1. It is known that Bid and Bax, which mediate mitochondrial membrane permeability, are substrates of calpains. Upon apoptotic stimulus, Bid is cleaved by calpains in the cytosol (27, 28). The truncated Bid then translocates to the mitochondria, generating pores and releasing cytochrome c. Similarly, Bax is cleaved by calpains mediating the release of cytochrome c (25, 26). Additionally, mitochondrial membrane permeabilization induces translocation of several molecules into the cytosol, including AIF, cytochrome c, Apaf-1, and the dATP complex.

Mammalian AIF is located in the inner mitochondrial intermembranous space (51). Upon apoptotic stimuli, the AIF is cleaved by calpains or cathepsins (29, 30) yielding a soluble apoptogenic protein released into the cytosol. The AIF then translocates to the nucleus, where it mediates chromatin condensation and DNA fragmentation (51). A functional role of calpain-1 in the ischemia-induced neuronal injury has been demonstrated using the siRNA-based knockdown approach (38). This study showed that calpain-1 cleaves mitochondrial AIF, an essential step for its translocation from mitochondria to cytosol. The knockdown of calpain-1 prevented ischemia-induced AIF translocation (38). Consistent with these findings, our results show that the release of AIF from the mitochondria into cytosol is reduced in the calpain-1 KO neurons exposed to oxidative stress (Fig. 7). Thus, at least under the settings of ischemia and oxidative stress, calpain-1 appears to play a functional role in the regulation of apoptosis through the release of AIF.

Under basal conditions, a fraction of AIF was detected in the cytosol. The calpain-1 KO neurons showed a relatively higher amount of AIF in the cytosol than the WT or heterozygous (Fig. 7A). The increased amount of AIF in the calpain-1 KO neurons under basal conditions could be a consequence of compensation by proteases such as calpain-2 and cathepsins. It is to be noted that regardless of the higher amount of AIF in the cytosol, the calpain-1 KO cells show relatively less activation of caspase-3 and apoptosis under basal conditions as compared with the WT and heterozygous neurons.

The release of cytochrome c, Apaf-1, and dATP complex into cytosol activates caspase-9. The activated caspase-9 further activates caspase-3 resulting in DNA fragmentation. Generally, the DNA fragmentation is induced by two pathways, a caspase-dependent pathway and a caspase-independent pathway. Our findings indicate that the DNA fragmentation is reduced in the calpain-1 KO neurons (Fig. 3). This observation suggests that neuronal apoptosis mediated by oxidative stress is at least in part mediated by calpain-1 under these conditions. Interestingly, the pan-calpain inhibitor MDL did not further exacerbate these events in the calpain-1 KO neurons. This finding suggests a prominent role of calpain-1 in apoptosis under these conditions. It appears unlikely that calpain-2 would be activated in the calpain-1 KO neurons, particularly when the calcium entry is significantly blocked. Based on these findings, we suggest that calpain-1 plays a more dominant role than calpain-2 in the regulation of neuronal apoptosis induced by oxidative stress.

The activated caspase-3 induces DNA fragmentation, which is a hallmark of apoptosis. The oxidative stress directly results in the cleavage of DNA because of the hydroxylation of guanine and the methylation of cytosine (2). Interestingly, the cleavage and activation of caspase-3 were significantly blocked in the calpain-1 KO neurons (Fig. 4). Under basal conditions, the cleavage of caspase-3 was equivalent in WT, heterozygous, and calpain-1 KO mice (Figs. 3C and 4B). However, the caspase-3 activity was less in calpain-1 KO (Fig. 4C). Similarly, the calcium entry (Fig. 5B) and mitochondrial membrane potential (Fig. 6) did not show any significant difference between these genotypes. The apoptotic cells, indicated as TUNEL-positive cells (Figs. 3, A and B, and 7C), were relatively less in calpain-1 KO. Therefore, it is reasonable to assume that calpain-1 may regulate the activity of caspases via the cleavage of caspase inhibitors. It is to be noted that when caspase-3 is bound to its physiological inhibitor, XIAP, it does not show any protease activity even upon its cleavage (52). In vitro, the stability of XIAP is under the regulatory control of calpains, which degrade XIAP to release its inhibitory effect on caspases (53). In calpain-1 KO, the cleavage of XIAP may be altered thus affecting the activity of caspase-3. The cleaved caspase-3 appears to be inactive in the KO, thus generating relatively less apoptotic cells in the calpain-1 KO under basal conditions. Activation of caspase-3 has been observed in many neurodegenerative disorders, including TBI (54, 55), and in the models of ischemia and Alzheimer disease (56). Whether the regulatory role of calpain-1 in the activation of caspase-3 is a common feature of neuronal diseases will be clarified by future studies.

Together, our in vivo and in vitro findings suggest that the targeted inhibition of calpain-1 can offer a promising therapeutic approach against both TBI and other neurodegenerative diseases in which oxidative stress plays a role. Historically, it has been technically challenging to design calpain-1-specific inhibitors mainly because of the similarity of its catalytic domain with calpain-2 and cysteine proteases. As an alternative strategy, it may be feasible to inhibit the translocation of calpain-1 into mitochondria, which is an essential step for the cleavage of AIF. Calpain-1 has been localized at the inner membrane space of mitochondria (38) and is mediated by the unique mitochondrial localization signal present at the N terminus (57). In con-
trast, this mitochondrial localization signal is not found in the calpain-2 sequence (57). Alternatively, technical improvements of gene therapy or siRNA approaches to specifically knock down or silence calpain-1 may be attempted in models of TBI and neurodegenerative diseases. In summary, our studies support a functional role of calpain-1 in apoptosis induced by oxidative stress and in apoptosis-induced neurodegeneration following TBI in vivo.

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