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Calpain 1 and Calpastatin Expression is Developmentally Regulated

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Abstract

Calpains and caspases are cysteine endopeptidases which share many similar substrates. Caspases are essential for caspase-dependent apoptotic death where calpains may play an augmentive role, while calpains are strongly implicated in necrotic cell death morphologies. Previous studies have demonstrated a down-regulation in the expression of many components of the caspase-dependent cell death pathway during CNS development. We therefore sought to determine if there is a corresponding upregulation of calpains. The major CNS calpains are the μ- and m-isoforms, composed of the unique 80 kDa calpain 1 and 2 subunits, respectively, and the shared 28 kDa small subunit. In rat brain, relative protein and mRNA levels of calpain 1, calpain 2, caspase 3, and the endogenous calpain inhibitor calpastatin, were evaluated using western blot and real-time RT-PCR. The developmental time points examined ranged from embryonic day 18 until postnatal day 90. Calpain 1 and calpastatin protein and mRNA levels were low at early developmental time points and increased dramatically by P30. Conversely, Caspase-3 expression was greatest at E18, and was rapidly downregulated rapidly down-regulated between by P30. Calpain 2 protein and mRNA levels were relatively constant throughout the E18-P90 age range examined. The inverse relationship of calpain 1 and caspase 3 levels during CNS development is consistent with the shift from caspase-dependent to caspase-independent cell death mechanisms following CNS injury in neonatal vs. adult rat brain.

Keywords

calcium; apoptosis; necrosis; cell death; injury

Introduction

Insults to the early postnatal brain such as traumatic brain injury and hypoxia-ischemia result in predominately apoptotic neuron death, whereas necrotic death is more prevalent in response to insults at later developmental time points (Bittigau, et al., 2004, Raghupathi, 2004, Sutton, et al., 1993, Werner and Engelhard, 2007, Zhu, et al., 2005). Caspases 3 ,7 and 8 are present at relatively high levels in the prenatal and early postnatal rat brain, but are abruptly

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downregulated within one month following birth (Madden, et al., 2007, Shimohama, et al., 2001, Yakovlev, et al., 2001). The decrease in caspase expression corresponds with the reduction in apoptotic susceptibility in the developing brain, but it is uncertain whether upregulation of other death-related proteases contributes to the increased susceptibility to necrotic death with brain maturation.

Calpains are calcium-activated cysteine proteases implicated in necrotic death (Liu, et al., 2004, Pang, et al., 2003, Syntichaki, et al., 2002, Wang, 2000). The best characterized, and the predominant calpains in the CNS, are the ubiquitous m- and μ-calpains (Goll, et al., 2003). These are heterodimers consisting of a unique 80 kDa large subunit (Calpain 1 and 2) and a common 28 kDa small subunit (Calpain Small Subunit 1, also referred to as Calpain 4) (Goll, et al., 2003, Suzuki, et al., 2004). Physiologic roles of calpains include cell motility and attachment, differentiation, signal transduction including cell survival pathways, membrane fusion, cell cycle progression, regulation of gene expression, and long term potentiation (Franco and Huttenlocher, 2005, Goll, et al., 2003). Many calpain substrates are similar to, or overlap with, those of caspase 3 (Wang, 2000), consistent with the hypothesis that calpains may also function as death-related proteases.

The purpose of this study was to evaluate the developmental regulation of the Calpain 1 and 2 large subunits, along with the endogenous calpain inhibitor, calpastatin, in the CNS. Previously, we and others observed that at least some of the calpain 1 is present in mitochondria (Badugu, et al., 2008, Cao, et al., 2007, Garcia, et al., 2005, Kar, et al., 2007, Kar, et al., 2008). Therefore, the developmental expression of calpain 1 in isolated rat brain mitochondria was also determined.

Materials and Methods

All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee. Rats greater than 15 days of age were euthanized by carbon dioxide inhalation followed by decapitation. Rats of age 15 days or less were euthanized by decapitation.

Brain homogenates

Brains were removed from embryonic day 18 (E18), postnatal day 5 (P5), P10, P15, P20, P30 and P90 Sprague-Dawley rats following euthanasia. The brain tissue was homogenized in four volumes of 50mM Tris-buffered saline (TBS), pH7.5, 10mM EDTA, 1mM AEBSF(4-[2 aminoethyl]-bensenesulfonyl fluoride, HCl), 0.1mM leupeptin and 1μM pepstatin. The homogenate was centrifuged at 13,000*g* for 20 min at 4°C. Protein content of the supernatant was determined by the BCA protein assay.

Mitochondria isolation

The mitochondrial isolation procedure from rat cortex was as described previously (Garcia, et al., 2005, Naga, et al., 2007) with slight modifications. Following euthanasia by CO_2 asphyxiation, rats were decapitated and the brains rapidly removed. The cortices were dissected, minced, and homogenized (10% w/v) in mitochondrial isolation buffer (MIB) (215 mM mannitol, 75 mM sucrose, 20 mM HEPES, 1 mM EGTA, and 1× complete protease inhibitor, pH adjusted to 7.2 with KOH) in a glass dounce homogenizer. After a 3 min spin at 1300*g*, 4 °C, the supernatant was collected. The pellet was resuspended in MIB and centrifuged again. The pooled supernatants were centrifuged at 13,000*g*, 10 min, 4 °C, and the pellet was resuspended in MIB and then mixed with an equal volume of 30% Percoll in isolation buffer. The resultant homogenate was layered on a discontinuous Percoll gradient with the bottom layer containing 40% Percoll solution in isolation buffer, followed by a 24% Percoll solution,

and finally the sample in 15% Percoll. The density gradients were centrifuged in a fixed angle SE-12 rotor at 30,400*g* for 10 min, 4 °C. Following centrifugation, fraction 3 was removed from the interface between the 24% and 40% Percoll, diluted in MIB, and centrifuged at 16,700*g* for 15 min, the pellet was washed and centrifuged at 13,000*g* for 10 min.

Western blot

The samples were mixed with one-half volume of concentrated (3×) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer and heated (100°C) for 10 min. Homogenates (80 μg protein/lane) or mitochondrial samples (30 μg protein/lane) were run on 10% Tris-Glycine gels and transferred to 0.45 μm nitrocellulose membranes. A pooled E18 homogenate was also added to each gel for between blot comparisons. The membranes were blocked in 5% (wt/vol) non-fat milk in 50 mM Tris–saline for 60 min at room temperature. After washing in TBS, the membranes were incubated with anti-caspase-3 (Cell Signaling Technology, Cat#9665), anti-calpain 1 (Abcam, Cat#ab28257, polyclonal antibody against N-terminal of calpain 1), anti-calpain 2 (Calbiochem, Cat#208737, monoclonal antibody against domain III), or anti-calpastatin (Chemicon, Cat#MAB3084) antibodies at room temperature overnight. After washing, the membranes were incubated with the species-appropriate IRDye Secondary Antibodies (LI-COR Bioscience) for 60 min at room temperature. Immunoreactive products were visualized and quantified using Odyssey (LI-COR Bioscience). Caspase 3 was detected as two bands of 32kDa and 29 kDa which were quantified together. After detection, the membranes were stripped and reprobed for μ-tubulin (Abcam, Cat#ab6046) as a loading control. For mitochondrial samples, mitochondrial HSP70 (Affinity Bioreagents, Cat#mA3-028) was probed as a loading control. Percent changes represent the ratio of different postnatal developmental time points to E18 expression values.

Real-time RT-PCR

Total RNA was isolated from rat brains using TRIzol Reagent (Invitrogen) and treated with DNAse1, RNAse free (Roche), and further purified using an RNeasy Mini Kit (Qiagen). Two micrograms of total RNA were used for cDNA synthesis using high-capacity cDNA reverse transcription kits (Applied Biosystems). PCR was performed on a StepOne Real-Time PCR System (Applied Biosystems) using TaqMan Gene Expression Assays (Rn00569689_m1 for calpain1, Rn00567422_m1 for calpain2, Rn00583952_m1 for calpastatin, Rn00563902_m1 for caspase 3, and 4352930E for 18S rRNA). For each real-time PCR run, 18s rRNA was assessed in parallel, and the expression value for each gene was calculated by normalization to 18s rRNA. Fold changes represent the ratio of different postnatal developmental time points to E18 expression values.

Results

In Western blots of rat brain homogenates, immunoreactivity of the 80 kDa Calpain 1 increased 75% from E18 to P90, with the increase being most rapid between P10 and P20 (Fig. 1A,B). Developmental changes in calpastatin expression were very similar to calpain 1, increasing 75% from E18 to P90. In contrast, calpain 2 levels were relatively constant in the late embryonic, postnatal, and adult rat brain. Levels of caspase 3 declined 85% from E18 to P90 (Fig. 1A,B).

The developmental regulation of calpain 1 levels within rat cortical mitochondria was similar to that observed for brain homogenates, being present at low levels at early postnatal time points and increasing substantially during postnatal development (Fig. 1C).

Calpain 1 and calpastatin mRNA levels increased by over 2.5 fold; and caspase 3 levels declined by 7.5 fold, in rat brain from E18 to P90 (Fig. 1D). Similar to the results observed for

protein levels, calpain 2 mRNA expression was relatively constant throughout the developmental time points examined (Fig. 1D).

Discussion

The results demonstrate that calpain 1 and calpastatin are upregulated during postnatal CNS development. Their protein and mRNA levels are inversely related to those of caspase-3, the developmental downregulation of which was observed previously (Madden, et al., 2007, Shimohama, et al., 2001, Yakovlev, et al., 2001). In contrast to the above proteins, the expression of calpain 2 is relatively constant in the late embryonic, postnatal, and adult rat brain.

With a postnatal increase in the levels of both calpain 1 and the inhibitor calpastatin, it is difficult to predict the net effect on calpain activity, which was not examined in the present study. Developmental profiles of calpain activities were previously examined in rat and rabbit brain, with contrasting results obtained. Simonson and colleagues (1985) observed a developmental decrease in calpain 1 and 2 activities in rat forebrain, decreased calpain 1 and steady calpain 2 in the hindbrain, with no change in calpastatin activity in forebrain or hindbrain. Blomgren and Karlsson (1989) found a postnatal increase in calpastatin activity in rabbit brain, consistent with the results of the present study. They also observed that net calpain activity increased shortly after birth and then declined. Chakrabarti et al. (1993) detected a postnatal increase in calpain 2 (m-calpain) activity, with a slight postnatal decline in calpain 1 (μ-calpain) activity, and did not examine calpastatin. Developmental changes in the protein levels of calpastatin and calpains 1 and 2 were previously examined by Zhu and colleagues (2005), who observed no change in calpain 1 and a slight decline in calpain 2 between postnatal days 5 and 60. The reason for the discrepancy between these findings and the present study is unknown. In cultured cortical and hippocampal neurons, calpain activity increased with time in culture, but this was associated with increased expression of NMDA receptors and not related to calpain 1 levels (Dong, et al., 2006).

The increased expression of calpain 1 and decreased expression of caspase 3 during brain development corresponds with a shift in cell death morphologies. Apoptotic neuron death is evident in the embryonic brain, where it contributes to neuronal development, but is difficult to detect in the adult CNS (de la Rosa and de Pablo, 2000, Yeo and Gautier, 2004). Following cerebral ischemia and hypoxia-ischemia in rats and mice, there is a progressive shift from apoptotic to necrotic morphologies and decreased caspase-3 activation with increasing postnatal age (Gill, et al., 2002, Hu, et al., 2000, Liu, et al., 2004, Zhu, et al., 2005). Bittigau and colleagues observed that the developing rat brain is particularly sensitive to apoptotic neurodegeneration following traumatic brain injury (Bittigau, et al., 2003, Bittigau, et al., 2004). In the mature brain, neurotrauma-induced neuron death is predominantly necrotic, although overlapping morphologies with characteristics of apoptosis and necrosis are also observed (Raghupathi, 2004). Following excitotoxin injection into the rat CNS, apoptotic morphologies are most prevalent at early developmental ages, while necrotic death is favored at more mature developmental stages (Portera-Cailliau, et al., 1997, Portera-Cailliau, et al., 1997).

In apoptosis, calpains play an augmentive role that is cell- and insult-dependent (Huang and Wang, 2001, Lu, et al., 2002). In necrotic or oncotic cell death, calpains play an essential role (Liu, et al., 2004, Pang, et al., 2003, Syntichaki, et al., 2002). Thus, the developmental shift in cell death mechanisms from caspase-dependent to caspase-independent involves not only the down-regulation of caspases and other apoptosis-related proteins, but also the upregulation of calpain 1. This shift in relative protein levels is maximal between P10 and P20, which corresponds to a time of synaptogenesis and proliferation of oligodendrocytes (Aghajanian and

Bloom, 1967, Eayrs and Goodhead, 1959, Sturrock, 1974). In contrast, caspase-3 levels are maximal in the embryonic rat brain, corresponding to a time of widespread programmed cell death (Blaschke, et al., 1996).

Although previously thought to be cytosolic, at least a portion of the cellular calpain 1 is located in the mitochondrial intermembrane space (Badugu, et al., 2008, Cao, et al., 2007, Garcia, et al., 2005). The developmental regulation of mitochondrial calpain 1 expression was similar to that observed in the whole brain homogenates, being expressed at low levels in the early postnatal period and then increasing rapidly. This corresponds to the rapid proliferation of rat brain mitochondria between P7 and P25, required for the increase in oxidative phosphorylation and increased synaptic activity (Erecinska, et al., 2004, Pysh, 1970). Apoptosis-inducing factor (AIF), which when released from mitochondria translocates to the nucleus to induce caspase-independent death, is a putative calpain 1 substrate (Cao, et al., 2007, Polster, et al., 2005) . We therefore hypothesized that AIF would be cleaved by mitochondrial calpain 1 (Badugu, et al., 2008, Garcia, et al., 2005), although a subsequent study failed to support this hypothesis (Joshi, et al., 2009). The function and substrates of mitochondrial calpain 1 remain to be determined.

The lack of change in calpain 2 levels during the developmental time points examined is also of interest. Calpain 2, the large subunit of m-calpain, has been implicated in cell motility, chromosome alignment during metaphase, and fusion of myoblasts during myogenesis (Franco and Huttenlocher, 2005, Honda, et al., 2008, Honda, et al., 2004). Calpain 2 is essential for normal embryonic development, as disruption of the *Capn2* gene results in embryonic lethality, while calpain–1 null mice are viable and fertile (Azam, et al., 2001, Dutt, et al., 2006). Calpain 2 is expressed at high levels in proliferating precursor cells, but is downregulated in postmitotoic cells (Raynaud, et al., 2008). In the mouse CNS, calpain 2 expression is downregulated by E14.5 (Raynaud, et al., 2008). The lack of change in calpain 2 expression during later CNS development suggests that calpain 1 may play a more prominent role within neurons.

Phenotypes resulting from calpain 1 knockout are relatively mild and include a reduction in platelet aggregation and clot retraction, and platelet accumulation of protein tyrosine phosphatase 1B (Azam, et al., 2001, Kuchay, et al., 2007). Additional physiologic roles of calpain 1 remain to be identified.

Mechanisms underlying the developmental regulation of calpains 1 and 2, calpastatin, and caspase 3 are not well-understood. The caspase-3 gene promoter lacks a TATA-box but contains several SP1 elements and an Ets-1 like motif (Liu, et al., 2002). Calpastatin has four putative promoter regions, each of which has one or more SP1 elements (Raynaud, et al., 2005). Although two TATA box regions are found in exon 14t of the bovine *CAST* gene, these are not thought to have transcriptional activity. Other motifs in the *CAST* promoter region include SRY, AP-1 GATA-1, and GATA-2 motifs (Raynaud, et al., 2005). The promoter regions for the genes encoding calpains 1 and 2, *CAPN1* and *CAPN2*, have not been characterized.

In summary, the developmental upregulation of calpain 1 and calpastatin, combined with the downregulation of caspase 3 and related proteins, is consistent with a shift in cell death morphologies following CNS insult. Following insult, strategies to minimize apoptotic death via caspase inhibition are therefore most effective in the infant brain while calpain inhibition may be more appropriate for treatment of CNS injury at later developmental stages. The results also suggest caution in extrapolating results from embryonic models of CNS injury, such as primary neuronal cultures, to the mature brain.

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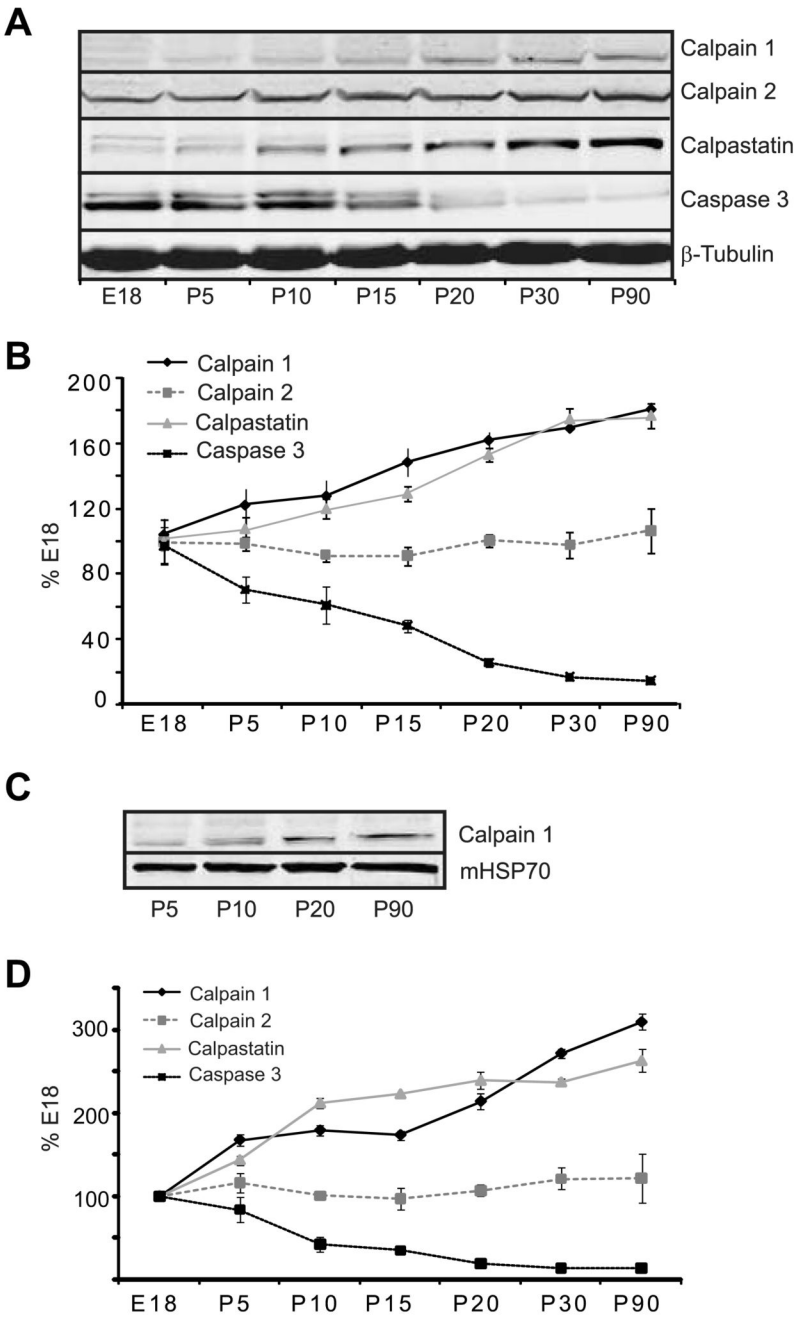

Figure 1.

Postnatal increase in Calpain 1 and Calpastatin protein and mRNA expression. In western blots of rat brain homogenates (Panel A) obtained from embryonic day 18 (E18) to postnatal day 90 (P90) animals, relative levels of calpain 1, calpain 2, calpastatin, and caspase 3 were examined. β-Tubulin was also examined as a loading control. Quantitative analysis of the western blots (Panel B) indicated that calpain 1 and calpastatin protein expression increased substantially between P10 and P30, compared with early developmental time points. Conversely, caspase-3 expression was greatest at the earliest time point examined (E18) and decreased dramatically during postnatal development. The results are expressed at the % of E18 levels, mean ± SEM, n=4. Calpain 2 levels were relatively constant throughout the embryonic, postnatal, and adult

time points examined. The developmental changes in calpain 1 expression in mitochondrial fractions from rat cerebral cortex (Panel C) were similar to those observed in the whole brain homogenates. Real-time RT-PCR revealed that calpain1 and calpastatin mRNAs (Panel D) exhibited similar expression profiles, being expressed at relatively low level in the embryonic brain and increasing rapidly by postnatal day 5-10. Calpain 2 mRNA levels were relatively similar at all stages of development . Caspase3 mRNA decreased substantially between P5 and P20, then remained low into adulthood. The results are expressed at the % of E18 levels, mean ± SEM, n=4.