Subcellular Localization and Duration of μ-Calpain and m-Calpain Activity After Traumatic Brain Injury in the Rat: A Casein Zymography Study


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Summary: Casein zymographic assays were performed to identify changes in μ-calpain and m-calpain activity in naive, sham-injured, and injured rat cortices at 15 minutes, 3 hours, 6 hours, and 24 hours after unilateral cortical impact brain injury. Cortical samples ipsilateral and contralateral to the site of injury were separated into cytosolic and total membrane fractions. Marked increases in μ-calpain activity in cytosolic fractions in the ipsilateral cortex occurred as early as 15 minutes, became maximal at 6 hours, and decreased at 24 hours to levels observed at 15 minutes after injury. A similar temporal profile of cytosolic μ-calpain activity in the contralateral cortex was observed, although the increases in the contralateral cortex were substantially lower than those in the ipsilateral cortex. Differences were also noted between cytosolic and total membrane fractions. The detection of a shift in μ-calpain activity to the total membrane fraction first occurred at 3 hours after traumatic brain injury and became maximal at 24 hours after traumatic brain injury. This shift in μ-calpain activity between the two fractions could be due to the redistribution of μ-calpain from the cytosol to the membrane. m-Calpain activity was detected only in cytosolic fractions. m-Calpain activity in cytosolic fractions did not differ significantly between ipsilateral and contralateral cortices, and increased in both cortices from 15 minutes to 6 hours after injury. Relative magnitudes of m-calpain versus μ-calpain activity in cytosolic fractions differed at different time points after injury. These studies suggest that traumatic brain injury can activate both calpain isoforms and that calpain activity is not restricted to sites of focal contusion and cell death at the site of impact injury but may represent a more global response to injury.

Trumatic brain injury (TBI) can result in excitotoxic consequences that have largely been attributed to pathological increases in intracellular calcium (Fineman et al., 1993; Nilsson et al., 1993; Shapira et al., 1990; Nadler et al., 1995), for reviews see: Hayes et al., 1992; McIntosh, 1996). Loss of calcium homeostasis can result in activation of calcium-dependent proteases, calpains, that may be one of the principle causes of pathology after TBI (for reviews see: Wang and Yuen, 1994; Hui and Wang, 1996; Kampfl et al., 1997). TBI produces significant degradation of all three major classes of cytoskeletal proteins, including microtubule-associated protein (MAP2), Tau et al., 1992; Hicks et al., 1995; Posmantur et al., 1995), intermediate filament proteins (i.e., low and high molecular weight neurofilament proteins; Posmantur et al., 1994, 1990a,b) and microfilaments (i.e., spectrin; Kampfl et al., 1996; Posmantur et al., 1990a,b; Sastmann et al., 1996a). These cytoskeletal proteins are all substrates for calpain proteolysis. Some studies of cytoskeletal proteolysis after TBI have reported the accumulation of calpain-specific breakdown products (Posmantur et al., 1994; Sastmann et al., 1995; Kampfl et al., 1996; Newcomb et al., 1997). Furthermore, inhibitions of calpains (and other proteases) have been shown to attenuate cytoskeletal protein loss (Posmantur et al., 1997) and provide behavioral protection (Sastmann et al., 1996).
Hayes et al., unpublished data) after experimental brain injury in mice. Finally, subcutaneous autoinjection of α-caspain, an event thought to accompany caspase activation, has been reported to occur between 10-30 minutes and 2-24 hours after TBI (Kampf et al., 1996). Despite a number of congent indirect lines of evidence for the role of caspase in pathological responses to TBI, no studies to date have provided direct evidence of increased caspase activity after injury. Moreover, no studies of central neuronal system injury have systematically compared changes in activity of the major caspase isoforms. The absence of such data may be attributable to difficulties in examining caspase activity in vivo partly due to the presence of the endogenous caspase inhibitor, caspase. Recently, a zymographic assay for caspases using non-denaturing caspase-containing polyacrylamide gels has been developed that circumvents this limitation (Roser et al., 1995). In addition, for technique allows for the differential and concurrent measurement of the two major isoforms of caspase, α-caspase and μ-caspase. The technique also provides the opportunity for analyzing protease activities in cytosolic and total membrane fractions, an important consideration because translocation of caspase may be a determinant of its attack on membrane-bound cytoskeletal protein targets (Saido et al., 1994). The present study represents the first application of this technique to in vivo studies of central nervous system injury. We report that TBI results in increases in μ-caspase and μ-caspase activity that occur as early as 15 minutes after injury and persist as long as 24 hours after injury. Increased cytosolic μ-caspase activity is associated with translocation of this isoform to the membranes, whereas increased μ-caspase activity remained in the cytosol.

**MATERIALS AND METHODS**

**Chemicals**

Dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethylene glycol (sodium salt) (EGTA), phenylmethylsulfonyl fluoride (PMSF), 4-(2-aminoethyl)-benzylamine (APEX), leupeptin, pepstatin and caspase were obtained from Sigma Chemical Company (St. Louis, MO). Tris base, glycine, sodium dodecyl sulfate, N,N,N',N'-tetraethyl glycylenlyleucyllythylenleucyllysine and Carboxyfluorescein were obtained from BioRad Laboratories (Heracles, CA, U.S.A.).

**Rat model of traumatic brain injury**

A controlled cortical impact device was used to induce TBI as previously described (Down et al., 1991). Briefly, adult male Sprague-Dawley rats (220 to 300 g) were anesthetized with 2% halothane in a 2:1 mixture of N2O/O2. After cranium exposure adjacent to the central sulcus, midway between lambda and bregma, injury was induced by impacting the right cortex (2.5-mm diameter) with a 6-mm diameter tip at a rate of 6 m/s. The injury produces focal contusion and necrosis in the ipsilateral cortex at the site of impact (Kampf et al., 1996; Postmann et al., 1996). In sham-injured animals underwent identical impact, but did not receive impact injury. These rats were not exposed to any surgical procedures.

**Assessment of caspase activity**

**Tissue homogenization.** All animals were deeply anesthetized with pentobarbital (100 mg/kg, intraperitoneally) and were decapitated at 15 minutes, 3 hours, 6 hours, and 24 hours (n = 3) after TBI or 1 hour after sham injury. As in Kampf et al. (1996), excess of both cortices (lateral and central to the injury) to the slices were removed using a 5-mm diameter stereotaxically calibrated cannula to 20 mm from the cranial bone. Tris-HCl (pH 7.4), 5 mM EDTA, 5 mM EGTA, 300 mM NaCl, 10 mM DTT, 0.5 mM PMSF, (1 μg/mL), APEX, 5 μg/mL leupeptin, and 15 strokes in a glass-Teflon homogenizer at 4°C, and centrifuged at 100,000 g for 40 minutes to obtain cytosol (supernatant) and total membrane (pellet) fractions (Ouwald et al., 1993). The pellets were resuspended in the same homogenization buffer and cleared with 1.0M sucrose with a 2:1 gauge needle by 15 passes. Protein content in samples was assessed by the micro-bicinchoninic acid (BCA) method (Pierce, Rockford, IL, U.S.A.). All samples were aliquoted and stored at −70°C until use.

**μ-Caspase isolation.** For μ-caspase isolation, we used a method of cell purification (Vito and Nixon, 1986). Five milliliters of rat blood was collected in 5 mL Tris saline buffer (TBS) [20 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5 μg/mL leupeptin] and centrifuged at 500 g for 10 minutes. Blood cells were removed in 5 mL of the TBS and then sonicated at 20 KHZ on ice for 5 minutes. After centrifugation at 11,000 g for 10 minutes at 4°C, the supernatant was mixed with precooled DE-52 cellulose and packed in a 15 × 2.5 column (BioRad). The column was eluted with Tris Buffer (TBS) [20 mM Tris-HCl (pH 7.6) buffer containing 5 mM MgCl2, 1 mM EDTA, 5 mM DTT, 5 μg/mL leupeptin] and then eluted with 5 mL of 150 mM NaCl in the TBS. The crude elution was used as the μ-caspase matrix.

**μ-Caspases.** The μ-caspase protein was purchased from Sigma.

**Caspase zymogram.** As has been previously described (Roser et al., 1995), casein (0.2%, weight/volume) was copolymerized in 12% (w/v) acrylamide separating gel (75 mM Tris-HCl, pH 8.8), 4% (w/v) acrylamide gel (300 mM Tris-HCl, pH 6.8) was used in the stacking gel. The casein gels were pre-run at 130 V for 2 hours at 4°C in a running buffer (25 mM Tris base, 192 mM glycine, 1 mM EDTA, 1 g/mL, and 1% (w/v) SDS, pH 8.3). One hundred fifty microliters of each sample was mixed with one fifth volume of sample buffer (125 mM Tris-HCl, pH 6.8), 20% glycerol, 2 mM EDTA, 2 mM mercaptoethanol, 0.004% (w/v) bromophenol blue, loaded in each well, and given electrophoresis at 20 V, 4°C for 16 to 18 hours. The gel was rinsed in immersion buffer (20 mM Tris-HCl, pH 7.5, 50 mM EDTA, 3 mM MgCl2, twice in the same immersion buffer at 32 ± 2°C for 24 hours. The gel was stained in 0.2% Coomassie blue for 2 hours and destained in a destaining solution (5% methanol, 3% acetic acid in distilled water) overnight. Coomassie image analysis software 5 was used to analyze the pictures on a Macintosh computer. Results were normalized as the optical density (arbitrary densitometric units) of the linear region (fitted band) divided by the protein amount for each sample run. A Polyfriction gels were used for quantification.

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Assessment of m-calpain protein levels

Western blotting. Because we had previously examined lev-
els of m-calpain in identical brain regions at the same time
points after cortical impact injury (Kampfl et al., 1996a), we
examined only levels of m-calpain protein. As described in
Pissinou et al. (1994), microdissected tissue was homoge-

Data analysis

Statistical analysis of the data was performed using an analy-
sis of variance with the Waller-Duncan K ratio to determine
group differences in Student's t-test. Significance was taken as
P < 0.05. Variance was expressed as SEM.

RESULTS

m-Calpain analysis

Densitometric analyses detected little m-calpain activ-
ity in naive rats and low levels of activity in sham-injured
rats (Fig. 1). Cortical impact injury resulted in significant in-
creases in m-calpain activity in cytosolic fractions from the
isplateral cortex that occurred as early as 15 minutes after
injury, peaked at 6 hours after injury, and remained above sham-injury levels at 24 hours after
injury (P < 0.05, compared to sham injury). Increases in
m-calpain activity in total membrane fractions from the
ipsilateral cortex were not detected until 3 hours and
were maximal at 24 hours after injury. In the contralat-
eral cortex, m-calpain activity was significantly elevated
in cytosolic fractions at 3 hours after injury, peaked at 6
hours after injury (P < 0.01 compared to sham injury) and
did not differ from sham-injury values by 24 hours after
injury (P > 0.05). Evidence of m-calpain activity was not
detectable in membrane fractions from the contralateral
cortex until 3 hours after injury and was maximal at 24
hours after injury. Levels of m-calpain activity in the
m-Calpain analysis:

Densitometric analysis detected no significant differences in m-calpain activity between naive and sham-injured rats (Fig. 2). Cortical impact injury resulted in significant increases in m-calpain activity in cytosolic fractions from 15 minutes to 6 hours after injury in the ipsilateral cortex (P < 0.01 compared to sham-injury). By 24 hours after injury, m-calpain activity in cytosolic fractions from the ipsilateral cortex was not significantly different from sham-injury values. In the contralateral cortex, m-calpain activity was significantly increased in cytosolic fractions only at 15 minutes after injury (P < 0.05 compared to sham injury). There was no detectable m-calpain activity in total membrane fractions. Levels of m-calpain activity in cytosolic fractions from the contralateral cortex were not significantly different from values observed in the cortex ipsilateral to injury.

Western blotting analyses detected prominent 80-kd immunoreactive bands in the absence of lower molecular weight bands, suggesting no autolysis or further processing of the parent protein (Fig. 3). There was no apparent change in m-calpain immunoreactivity in cytosolic or total membrane fractions from samples taken at various times after TBI. Immunoreactivity to m-calpain was more apparent in cytosolic than in total membrane fractions and did not differ in samples taken from cortices ipsilateral and contralateral to the site of injury.

Comparison of μ-calpain m-calpain activity

Because incubation buffers for calpain isoforms require different pH values for optimal detection of calpain activity (pH 7.5 for μ-calpain; pH 7.3 for m-calpain),

FIG. 3. Western blots of μ-calpain protein in cytosolic (supernatant) and total membrane (pellet) fractions in ipsilateral and contralateral cortices after experimental traumatic brain injury. Analysis detected prominent 80-kd immunoreactive bands to m-calpain that were more evident in cytosolic than total membrane fractions. There was no apparent change in μ-calpain immunoreactivity from naive (lanes 1, 2) to sham injured (lanes 3, 4) and injured cortices at 15 minutes (lanes 6, 7), 3 hours (lanes 4, 10), and 6 hours (lanes 8, 11), and 24 hours (lanes 6, 12) after injury. (A) Densitometric analysis of μ-calpain activity in cytosolic supernatant fractions from cortical samples ipsilateral and contralateral to the site of injury. Cortical injury produced significant increases in μ-calpain activity in cytosolic fractions from 15 minutes to 6 hours after injury in the ipsilateral cortex. In the contralateral cortex, μ-calpain activity was increased in the cytosolic fractions only at 15 minutes after injury (P < 0.05; **, P < 0.01; *** P < 0.001). See text for details.
experiments routinely used separate gels to assess m-calpain and μ-calpain activity. The use of separate gels prevents direct comparisons of activity of μ-calpain and m-calpain. However, Table 1 summarizes the ratios of differences in optical densities for cytosolic fractions of samples assaying μ-calpain and m-calpain activity after TBI. These ratios represent an indirect assessment of the relative magnitude of m-calpain versus μ-calpain activity in cytosolic fractions at different time points after injury.

DISCUSSION

The present study represents the first application of casein zymography to study calpain activity after experimental brain injury in vivo. These observations also provide the first systematic comparisons of changes in the two major calpain isoforms after injury to the central nervous system. We observed significant increases in cortical μ-calpain activity as early as 15 minutes after cortical impact injury that persisted for up to 24 hours. Separate examinations of cytosolic and total membrane fractions provided evidence for translocation of calpain activity from the cytosol to membrane by 3 hours after injury. In contrast, TBI produced relatively less activation of m-calpain detectable only in cytosolic fractions and which persisted no longer than 6 hours.

Our data on the temporal profile of μ-calpain activation using casein zymography show both similarities and differences to previously published observations on μ-calpain activation after TBI. Kampfl et al. (1996), using concurrent assessments of μ-calpain autolysis and accumulation of calpain-specific breakdown products to α-spectrin, concluded that μ-calpain activation after cortical impact injury occurred within 15 minutes, but returned to control levels within 24 to 48 hours after injury. The present data are in general agreement with an early onset of μ-calpain activation at the site of injury. Although Sastrean et al. (1996), did not detect calpainspecific breakdown products to α-spectrin at 30 minutes after injury, their studies used a different model (lateral fluid percussion) of TBI. The data presented here also indicate that μ-calpain activation may persist beyond the 24-hour duration suggested by studies of μ-calpain autolysis (Kampfl et al., 1996). This difference may be attributable to differences in mechanical injury magnitude in different experiments and/or differences in the sensitivities of casein zymography and μ-calpain autolysis to detect changes in calpain activity. Some reports have suggested that μ-calpain does not require autolysis for activation (Cong et al., 1989; Edmonds et al., 1991; Zhang et al., 1996; Guttman et al., 1997).

This study provides the first evidence of μ-calpain activation after TBI. As observed for μ-calpain, the data suggest rapid activation of m-calpain. Moreover, μ-calpain activation persisted for 6 hours after injury, a substantially brief period than μ-calpain activity which persisted for 24 hours. Although μ-calpain has micromolar sensitivity to calcium activation while m-calpain has a millimolar sensitivity to calpain activation (Hokosho et al., 1986), the relative differences in m-calpain activity and μ-calpain activity are not an artifact because the buffer used in these studies had sufficient Ca** (3.0 mmol/L) to fully activate both m-calpain and μ-calpain. The preferential activation of μ-calpain over m-calpain is also surprising in view of reports that the total amount of m-calpain exceeds by one to two orders of magnitude the amount of μ-calpain (Kuwashima et al., 1988), and similar profiles have been published for the content of the two isoforms in rat brain membranes (Siman et al., 1983).

The present study provides evidence that calpain activation was substantially higher at the site of cortical impact injury, a region associated with focal contusion and necrosis. The rapid onset of both μ-calpain and m-calpain activation, especially at the site of impact-induced focal contusion and necrosis, suggests that calpain activation may play an important role in cortical neuronal degeneration and even precede the full expression of evolutionary histopathologic changes characteristic of cortical impact injury (see Kampfl et al., 1996). Our study also provides evidence of μ-calpain and m-calpain activation in the contralateral cortex, a region not associated with focal contusions in this model system. Calpain specific breakdown products to α-spectrin have also been detected in the contralateral cortex after cortical impact injury (Posmann et al., 1997), and recent work in our laboratory has shown evidence of diffuse histopathology and cell death in the contralateral cortex.

| TABLE 1. Ratios of m-calpain versus μ-calpain activation after injury |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | Contralateral, cytosol       | Bilateral, cytosol           |                |
|                             | μ-Calpain | m-Calpain | Ratio (μg/μg) | μ-Calpain | m-Calpain | Ratio (μg/μg) |
| Naive                      | 0.0       | 0.6       | 1.0           | 0.0       | 0.06      | 1.0           |
| Sham-operated              | 0.08      | 0.11      | 1.1           | 0.13      | 0.13      | 1.0           |
| Injury: 15 min.            | 0.14      | 0.28      | 1.7           | 0.36      | 0.42      | 1.2           |
| Injury: 3 hours            | 0.25      | 0.26      | 1.0           | 0.47      | 0.29      | 1.6           |
| Injury: 6 hours            | 0.34      | 0.29      | 1.1           | 0.84      | 0.30      | 2.8           |
| Injury: 24 hours           | 0.12      | 0.16      | 1.2           | 0.40      | 0.18      | 0.5           |


1.0 πcalpain
after injury (Newcomb et al., 1997). However, Kampf et al. did not detect evidence of μ-calpain autolysis or ac-
cummulation of calpain-specific breakdown products to α-spectrin in the contralateral cortex after cortical impact
injury, an observation possibly attributable to use of a lower injury level than used in the present study. In any
case, our data support the possibility that calpain activa-
tion can extend into brain regions not associated with
focal contusions. This interpretation is consistent with
reports of degradation of cytoskeletal proteins in regions
distant from the site of cortical impact or lateral fluid
perfusion injury (Posmaat et al., 1996a; 1997; Saat-
mann et al., 1996; Newcomb et al., 1997).
Cerebral autolysis follows concurrent analyses of
both cytosolic and total membrane fractions. μ-Calpain
activity in the cytosolic fraction is increased in the cell
membrane. Saida et al. have argued that the pre-
autolysis form of μ-calpain migrates from the cytosol to
membranes where it becomes activated by autolysis
(e.g., Saida et al., 1993). Kampf et al. (1996) reported
that autolysis of μ-calpain after cortical impact injury
was well detectable in pellet fractions. Although our
data suggest that substantial activation of μ-calpain
occurs in the cytosolic fraction, there was evidence of
later translocation to the membrane-associated with more
prolonged calpain activation. The detection of μ-calpain
activity in cytosolic rather than total membrane fractions
is consistent with previous reports of cytosolic localiza-
tion of μ-calpain, although μ-calpain has also been de-
tected in purified myelin (Chakrabarti et al., 1996).
While Western blots in this study detected μ-calpain in
total membrane fractions, μ-calpain remained preferen-
tially in cytosolic fractions after TBI.
Our studies did not directly examine mechanisms me-
diating calpain activation after TBI. However, it is un-
likely that observed increases in μ-calpain activity are
due to increased amounts of μ-calpain in the protein
samples because previous studies by Kampf et al. (1996)
did not detect increased levels of μ-calpain at similar
time points after cortical impact injury. Similarly, in-
creases in μ-calpain activity are not related to an in-
creased amount of protein because Western blotting did
not detect any changes in μ-calpain immunoreactivity. It
is also unlikely that changes in calpain-calpastatin inter-
actions influence detection of calpain in the casein
zymogram because the method eliminates interactions be-
tween calpain and its endogenous inhibitor, calpastatin
(Raser et al., 1995). Moreover, increases in calpain ac-
tivity measured by casein zymography in this current
study are not likely due to calpain autolysis because un-
autolysed calpain would also be activated in the devel-
oped procedure. Although a clear autolyzed μ-calpain
form in casein zymography has been detected only from
cultured cells under maximal pathological conditions
(Raser et al., 1995), past and current in vitro studies in our
laboratory for unknown reasons have yet to detect the
shift in electrophoretic mobility associated with the au-
tolysed μ-calpain form (unpublished observations,
1996). Our in vitro data sampled calpain activity up to 24
hours after injury. More prolonged activation of calpain
in vivo could ultimately reduce activity of the enzyme.
For example, in vitro studies have suggested that lysates
contain calpain pools which have undergone sus-
tained activation would be predicted to contain less ca-
sein hydrolysis because the autolysed form would be unstable and undergo further degradation (Raser et al.,
1995). A probable cause of the increased casein hydro-
dysis in samples obtained after TBI may be due to post-
translational changes in calpain including interaction
with phospholipids or activator proteins that could en-
hance calpain activity (Saida et al., 1992; Chakrabarti
et al., 1996). Alternatively, nonactivated calpains (μ- and
μ) may be compartmentalized in resting neurons and
become accessible to calcium-dependent autolytic activ-
tion only in injured neurons or protein-separating tech-
niques. Our Western blotting also did not detect autolysed
degradation products to μ-calpain (Fig. 3). Although au-
tolysis of μ-calpain occurs both in m vitro preparation
and in cultured cells when m-calpain is activated, little
is known about m-calpain autolysis in vivo, and no studies
have addressed m-calpain autolysis after neuronal injury
(Nishimura and Goll, 1991; Wang et al., 1996; Nutt et
al., 1996).
In conclusion, the present study has provided strong
evidence that cortical impact injury results in rapid and
sustained increases in calpain activity. TBI also produces
relatively greater and more sustained increases in μ-
calpain activity than in μ-calpain activity. Future studies
need to examine mechanisms mediating calpain activity
after TBI. Because calpain activation has been impli-
cated in other brain regions such as the hippocampus
and thalamus (Posmaat et al., 1996a; Saatman et al.,
1996), additional brain regions should be examined.

Acknowledgement: The authors wish to thank Kim McBrayer for typing and editing this manuscript.

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