Activation of calpains, calpastatin and spectrin cleavage in the brain during the pathology of fatal murine cerebral malaria

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Abstract

Neuronal calpains appear to be activated uncontrollably by sustained elevation of cytosolic calcium levels under pathological conditions as well as neurodegenerative diseases. In the present study, we have characterized calpain activation in cytosolic extract of mice cerebral cortex and cerebellum using an experimental model of fatal murine cerebral malaria (FMCM). Pathology of FMCM resulted in the increase in activity of calpains in both cerebral cortex and cerebellum. Western blot analysis revealed an increase in the levels of μ-calpain (calpain-1) in the cytosolic fraction of infected cerebral cortex and cerebellum although a decrease in the level of μ-calpain was observed in the cytosolic fraction of infected cerebral and cerebral cortex. Calpain activation was further confirmed by monitoring the formation of calpain-specific spectrin breakdown products (SBDP). Protease-specific SBDP revealed the formation of calpain-generated 150 kDa product in the infected cerebral cortex and cerebellum. The specific signature fragment of calpain activation and spectrin breakdown after Plasmodium berghei ANKA infection provide a strong evidence of the role of calpains during the cell death in cerebral cortex and cerebellum. Given the role of calpains in neurodegeneration and cell death, our results strongly suggest that calpains are important mediators of cell injury and neurological sequelae associated with FMCM.

Keywords: Calpains; Fatal murine cerebral malaria (FMCM); Spectrin; Plasmodium berghei ANKA; Neurodegeneration; Neurological sequelae

1. Introduction

Calpains are cytoplasmatic calcium-dependent cysteine proteases (EC3.4.22.17) with two major isoforms (μ- and m-calpains also known as calpain-1 and calpain-2) that are ubiquitously expressed in mammalian cells (Sorimachi et al., 1997). The term μ-calpain and m-calpain refers to the micromolar (50 μM) and millimolar (0.2–1 mM) calcium requirement for the activity (Suzuki et al., 1995). Both enzymes are heterodimers, consisting of a large 80 kDa catalytic subunit that shares 55–65% sequence homology between the two proteases and a small identical 30 kDa regulatory subunit (Sorimachi et al., 1997). The activity of these proteases is tightly regulated by a highly specific endogenous inhibitor named calpastatin. Many putative calpain substrates are cytoskeletal proteins especially those involved in cytoskeletal/plasma membrane interactions, kinases and phosphatases, membrane-associated proteins including some receptors and ion-channel proteins and some transcription factors (Croall and DeMartino, 1991; Darrel et al., 2003). How calpain activity is regulated in cells is still unclear but, calpains ostensibly participate in a variety of cellular processes including remodeling of cytoskeletal/membrane attachments, different signal transduction pathways, cell cycle, regulation of gene expression, neuronal long-term potentiation and apoptosis (Darrel et al., 2003). However, neuronal calpains appear to be activated uncontrollably by sustained elevation of cytosolic calcium levels under pathological conditions, such as ischemia and spinal cord injury (SCI) (Banik et al., 1997) as well as in neurodegenerative diseases (Emil et al., 2002) like Alzheimer’s disease (Tsujii et al., 1998), Parkinson’s disease (Mouatt-Prigent et al., 1996) and amyotrophic lateral sclerosis (Ueyama et al., 1998) and also by ethanol consumption (Rajgopal and Vemuri, 2002).

Cerebral malaria (CM) is the life-threatening complication of Plasmodium falciparum infection in humans, responsible for more than one million deaths annually (Miller et al., 1994; Turner, 1997). Cerebral dysfunction becomes evident through a variety of symptoms, including extreme lethargy and febrile convulsions and can progress to coma and death in...
approximately 20% cases (Naili et al., 1997). Although a number of studies have described the neurological complications of human CM, the pathogenesis remains controversial. An experimental murine model, induced by the infection of CBA mice with *Plasmodium berghei* ANKA, has been used to further clarify the pathogenesis of CM apart from post mortem brain studies. Studies with this model have suggested that fatal murine cerebral malaria (FMCM) is either an immunopathological process involving CD4+ T lymphocytes, monocytes and cytokines or a combination of an immunopathological process together with the mechanical blockage of micro vessels by the monocytes and infected erythrocytes (Naili et al., 1997). The pathologic features of fatal stage include monocyte adhesion to cerebral vascular endothelial cells, edema and petechial hemorrhages in the central nervous system (CNS). All these features have been reported in the human post mortem brain tissue. Further, the pathology of FMCM is known to manifest both necrotic and apoptotic modes of cell death (Kumar and Babu, 2002; Kumar et al., 2003). Though the events central to tumor necrosis factor (TNF) and interleukin are activated both necrotic and apoptotic modes of cell death (Kumar and Babu, 2002). Homogenization was carried out in ice-cold isolation buffer (0.32 M sucrose, 10 mM Tris–HCl buffer (pH 7.2), 1 mM EDTA, 1 mM EGTA, and 1.5 mM MgCl2, 1 mM PMSE, 2 μg/ml leupeptin, 2 μg/ml aprotinin) and cellular fractions were prepared by centrifugation. For whole tissue lysate the cerebral cortex and cerebellum were homogenized in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% deoxycholate, 1% TritonX-100, 10% glycerol, 2 mM EDTA, 1 mM PMSE, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 20 mM sodium fluoride). Cell lysate was centrifuged at 14,000 × g for 15 min and the supernatant was taken as the whole tissue lysate. Protein content in the cellular fractions was quantified by the method of Bradford (1976).

### 2. Western immunoblotting

Fifty micrograms of cytosolic/whole tissue protein was separated by reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), and transferred onto nitrocellulose membrane overnight at 30 V in Towbin buffer (25 mM Tris–HCl buffer (pH 8.3), 192 mM glycine, 20% methanol). The non-specific binding sites were blocked with 5% (w/v) non-fat milk for 2 h. The blots were probed with primary antibodies. The primary antibodies were 1:200 dilution of mouse monoclonal antibody against μ-calpain, 1:500 dilution of rabbit polyclonal antibody against m-calpain (these two antibodies are gift from Dr. Panaiyur S. Mohan, Nathan Kline Institute, NY, USA), 1:1000 dilution of mouse anti-calpastatin (RDI research diagnostics, NJ, USA), 1:1000 dilution of rabbit polyclonal antibody against calpain spectrin breakdown products (SBDP) of α-spectrin (Rajgopal, University of Kentucky). The blots were further processed with either horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody. The protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL, USA) or TMB/H2O2 for localization (Bangalore Genei, India).

### 2.4. Calpain activity

Calpain activity was measured using azocasein (Sigma–Aldrich) as substrate (Takeuchi et al., 1992) at a final concentration 0.6% in 1.0 ml of reaction mixture containing 0.02% β-mercaptoethanol, 100 mM Tris–acetate buffer (pH 7.5), 10 mM KCl, 5 mM CaCl2 and 50 μg of enzyme solution. The control tubes were treated the same way except CaCl2 was replaced by EGTA (5 mM). The reaction was incubated for 30 min at 37 °C and was stopped by the addition of 0.4 ml of 20% trichloro acetic acid and left on ice for 30 min. The samples were centrifuged at 6000 rpm for 1 h and absorbance of supernatant was read at 366 nm using Shimadzu spectrophotometer. Calpain activity was indicated by an increase in the absorbance values compared to control. One unit of enzyme activity is defined as the amount of the enzyme that increases absorbance by one unit at 366 nm under the specific incubation conditions used.

### 2.5. Calpain-1 immunohistochemistry

Infected and control mice brain was perfused first with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4 and were paraffin embedded. Mouse brain was sectioned horizontally (5 μm) on the automated rotary microtome (Leica) and deparaffinized in xylene. Antigen retrieval was carried out by microwaving sections in 10 mM citrate buffer, pH 6.0, for 1 min at full power followed by 9 min at medium power. Blocking was carried out in

### 2. Experimental procedures

#### 2.1. Induction of cerebral malaria in mice

All the protocols followed for the use of animal experimentation were approved by the institutional as well as national ethical committee guidelines (CPCSEA, 2003). Six to eight-week-old Swiss albino mice of either sex (~20 g body weight, n = 4 per each group) were inoculated intraperitoneally with 10⁸ parasitized red blood cells, suspended in 200 μl of phosphate-buffered saline (PBS; pH 7.4). Uninfected mice of same age and sex were used as negative controls (n = 4). The animals infected with *P. berghei* ANKA strain showed behavioral changes around day 5 after inoculation followed by cerebral symptoms like paralysis, hemiplegia, convulsions and coma eventually leading to death of the animal. The parasitaemia was monitored by preparing periodic blood smears from day 3 of parasite inoculation and was typically between 40 and 50% at the time of sacrifice. The duration between parasite inoculation and sacrifice of animals was approximately 14–16 days.

#### 2.2. Sub-cellular fractionation of brain

Brain was dissected from control and cerebral malaria-infected animals after 7th, 10th, 12th and terminally ill after decapitation and stored at ~80 °C until use. Cerebral cortex and cerebellum regions were separated and cellular fractions (cytosol, membrane) and whole tissue lysate were prepared essentially as described earlier (Kumar and Babu, 2002). Homogenization was carried out in ice-cold isolation buffer (0.32 M sucrose, 10 mM Tris–HCl buffer (pH 7.2), 1 mM EDTA, 1 mM EGTA, and 1.5 mM MgCl2, 1 mM PMSE, 2 μg/ml leupeptin, 2 μg/ml aprotinin) and cellular fractions were prepared by centrifugation. For whole tissue lysate the cerebral cortex and cerebellum were homogenized in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% deoxycholate, 1% TritonX-100, 10% glycerol, 2 mM EDTA, 1 mM PMSE, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 20 mM sodium fluoride). Cell lysate was centrifuged at 14,000 × g for 15 min and the supernatant was taken as the whole tissue lysate. Protein content in the cellular fractions was quantified by the method of Bradford (1976).

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10% normal goat serum in PBS for 1 h at room temperature in a humidified chamber. Primary antibody (mouse monoclonal antibody against μ-calpain) was diluted 1:100 in blocking solution and incubated for overnight at 4°C. Peroxidase-conjugated anti-rabbit secondary antibody (1:250) was used for incubation time of 1 h at room temperature followed by PBS washes (3 × 5 min each). DAB at a final concentration of 0.25 mg/ml with 0.05% H₂O₂ in PBS was used for developing till the section turns brown (typically for 3–5 min at RT). Sections were counter stained with methyl green for 10 s. Sections were then washed with dH₂O followed by dehydration in graded ethanol and xylene and coverslipped with DPX mount.

2.6. Data analysis

Data are reported as mean ± S.E.M. of n experiments. All parameters were compared using a two-tailed Student’s t-test between infected and control animals. A level of \( P < 0.01 \) was considered statistically significant and was determined using Sigma plot 2000 for windows version 6.00, SPSS Inc., IL, USA.

3. Results

Pathology of FMCM resulted in the appearance of μ-calpain protein levels in the infected cerebral cortex and cerebellum (Fig. 1). Expression of μ-calpain protein levels was checked at various time intervals (Fig. 1), which clearly resulted in the expression of μ-calpain from day 7 onwards but expression was at its peak when the animals were terminally ill. Hence for other proteins we have taken terminally ill animals. Decreased level of m-calpain protein was observed in the cytosolic fraction of infected cerebral cortex as well as cerebellum when compared to control samples (Fig. 2a). An increased level of calpastatin protein was observed in the infected samples (Fig. 2b). Further to substantiate our results we performed calpain activity in the whole tissue lysate. Increased calpain activity in both infected cerebral cortex and cerebellum was observed (Fig. 3). Significant basal calpain activity was observed in the control cerebral cortex and cerebellum. To determine whether the calpain was indeed activated, we examined the cleavage of a well-characterized calpain substrate non-erythroid α-spectrin (also known as α-fodrin) (Fig. 4). In infected cerebral cortex and cerebellum α-spectrin (280 kDa) was cleaved by endogenous calpain to produce characteristic 150 kDa (SBDP150) as well as the loss of the intact α-spectrin, as reported previously (Roy et al., 1993; Saido et al., 1994). Significant increase in the levels of full-length α-spectrin was observed in the infected cerebral cortex, whereas no change was observed between the control and infected cerebellum. The antibody recognizes the calpain-specific cleaved fragment, the levels of which were observed only in the infected cerebral cortex and cerebellum. In these experiments α-tubulin was used as an internal control of protein being loaded onto SDS–PAGE. Calpain-1 immunoreactivity using mouse monoclonal antibody against μ-calpain, revealed more immunoreactive calpain-1 cells in the infected cerebral cortex and cerebellum (Fig. 5b and e). Though some calpain-1 immunoreactivity was also found in the control brain, which can be accounted for the basal levels of calpain-1 in the normal brain (Fig. 5a and d). Moreover, calpain-1 staining was clearly localized in the cytosol as well as other components of the neural cells (Fig. 5c and f). Our results show that calpains were indeed activated upon Plasmodium berghei ANKA infection in mice brain.

4. Discussion

Recent studies have focused on the pathological significance of calcium accumulation in the CNS following cerebral ischemia, SCI and traumatic brain injury (TBI). Disturbances in neuronal calcium homeostasis may result in the activation of several calcium-sensitive enzymes, including lipases, kinases, phosphatases and proteases. One potential pathogenic event in a number of acute CNS results, including neuronal ischemia, TBI, is the activation of the calpains (Kampfli et al., 1997). Intracellular Ca²⁺ increased in ischemic areas and MAP2 and α-spectrin are degraded in animal models of cerebral ischemia with the calpain-specific 150 kDa α-spectrin fragment appearing after ischemia in rat brains. The rate of expansion of the area of α-spectrin degradation is reduced by synthetic calpain inhibitors A275 and A295. Calpastatin is degraded by calpain to a 50 kDa membrane-bound polypeptide in ischemic brain (Blomgren et al., 1999). Tissue damage in the ischemic areas involves both apoptosis and necrosis and the calpains participate in both the processes (Darrel et al., 2003). Calpain activation and α-spectrin cleavage has been shown in rat brain by ethanol (Rajgopal and Vemuri, 2002). The putative role of calpains in various pathologies has been the subject of several reviews (Huang and Wang, 2001).

In the present study, increased total calpain activity was observed in the cerebral cortex and cerebellum upon Plasmodium berghei ANKA infection, which is in relevance with the increased calpain levels in the infected cortex and cerebellum. Moreover, increase in the expression levels of μ-calpain was observed from day 7 post infection. However, terminally ill animals showed highest levels. Therefore, we have chosen terminally ill animals for all other experiments. Such an increase in the

![Fig. 1. Immunoblot analysis of calpain-1 in cytosolic extracts of control and infected mice at various time intervals. Equal amount of protein was electrophoresed by SDS–PAGE and transferred to nitrocellulose membrane and probed with primary antibody to calpain-1. The result is representative of four independent experiments with similar results. The lanes—C1: control cerebral cortex; 7I1, 10I1, 12I1, T1I, T1I: infected cerebral cortex at 7th, 10th, 12th and terminally ill animals (T1I, T1I) and C2: control cerebellum; 7I2, 10I2, 12I2, T12, T12: infected cerebellum at 7th, 10th, 12th and terminally ill animals (T12, T12).](image)
Calpain activity might involve autoproteolysis of either of the calpain subunits (Elce et al., 1997). Hence, a decrease in the m-calpain protein levels was observed in the infected cerebral cortex and cerebellum that might be due to increased autoproteolysis of m-calpain upon its activation. According to earlier studies (Elce et al., 1997), the physiological importance of autolysis is probably that, it generates forms of active calpain with short half-lives resulting from their lower [Ca$^{2+}$]$_{0.5}$, their stability, and their further autodegradation, thus limiting the duration of calpain activity in vivo. Moreover, calpains are known to interact with a variety of intracellular substrates at both cytosolic and membrane sites (Saido et al., 1994), hence, examination of the sub-cellular distribution of calpains during injury/infection might indicate their site of action. Studies by Ostwald et al. (1994) reported similar distribution of calpain activity in normal rabbit hippocampal cells as well as calpain translocation after hypoxia. We examined m-calpain protein level in the cytosolic fraction as well as membrane fraction. We observed no change in the membrane factions. Hence, a decrease in m-calpain protein level might be due to some other reason.

Calpastatin is cleaved with oxidative stress. It is well known that calpastatin serves not only as the endogenous calpain inhibitor but also as a substrate for calpain at the cell membrane (Blomgren et al., 1999). Upon P. berghei ANKA infection, the levels of calpastatin appeared to be increased in the infected cerebral cortex and cerebellum. The affinity of the calpastatin is greater for the autocatalyzed calpain form than the intact native form (Croall and DeMartino, 1991). Calpastatin is the preferred substrate to calpains, more readily cleaved than fodrin, possibly because calpains complex with calpastatin before they have a chance to bind to fodrin and other membrane-associated substrates (Blomgren et al., 1999). However, calpastatin binding does not prevent the intramolecular, autolytic activation of calpains, and if the stimulus becomes stronger, the protease will degrade its inhibitor and then go onto cleave other substrates. Moreover, several reports have recently appeared demonstrating degradation of calpastatin by caspases also (Blomgren et al., 1999; Neumar et al., 2003). We have shown the increase in the activity and protein levels of caspase-3 [communicated data] upon P. berghei ANKA infection. Hence calpastatin might be cleaved by both caspases as well as calpains.

Among the various substrate proteins for calpain, fodrin has attracted a number of researchers, interested in the physiology...
and pathology of brain functions, since this major cytoskeletal protein was the first to be described to undergo calpain-catalyzed protein lysis both in long-term potentiation and post ischemic degeneration (Saido et al., 1993). In the present study we visualized the formation of calpain-specific cleaved fragment (SBDP150) in the infected cerebral cortex and cerebellum that is in agreement with the increased calpain levels and activity in the infected samples. The specificities of \(\mu\)- and \(m\)-calpain are very similar if not identical (Li et al., 2004; Darrel et al., 2003). \(\alpha\)-Spectrin degradation and the resultant disruption of cytoskeletal network might be necessary steps in the expression of apoptosis. It has been reported that fragmented \(\alpha\)-spectrin lacks the ability to interact with actin (Rathna et al., 1996). Thus \(\alpha\)-spectrin cleavage by calpain can potentially lead to cytoskeletal derangement. It is also of interest to point out that \(\alpha\)-spectrin binds to ankyrin that contains a cell death domain (similar to those found in TNF and Fas/Apo1) (Rathna et al., 1996). Thus calpains participate in apoptosis by exerting their effects on their target proteins.

In the present study, the levels of calpain-specific cleaved fragment drastically increased in the infected cerebral cortex and cerebellum indicates that the calpains are indeed activated. The observation of fodrin proteolysis described here implies that other calpain substrates also may have undergone proteolysis following calpain activation upon \(P.\) berghei \(ANKA\) infection. Earlier, from our laboratory a decrease in the activity and levels of PKC in cytosol has been reported in the present model (Kumar et al., 2003). The PKC-\(\alpha\), PKC-\(\beta I\), PKC-\(\beta II\) and PKC-\(\gamma\) isoforms are all rapidly cleaved from 82 kDa polypeptide to 45–49 kDa fragments by the calpains (Kishimoto et al., 1989). Activation of calpains in the present study is in relevance with the decreased levels and activity of PKC (Kumar et al., 2003) in this model. Recent data suggest that calpains are a key mediator of p53 induction and consequent caspase-dependent neuronal death due to DNA damage (Sedarous et al., 2003). We observed p53 induction (Kumar and Babu, 2002) and caspase activation [communicated] in the same model.

The results of the present study provide strong evidence that calpains are active components in the pathology of FMCM. However, each isoforms vary in the concentrations and levels of activation with the progression of pathology. The precise consequences of spectrin breakdown in terms of cytoskeletal damage that converges into cell death due to the activation of calpains in FMCM remain to be determined. Calpain activation by elevated intracellular calcium levels can induce proteolytic modifications in a number of proteins associated with multiple signaling cascades for mediating apoptosis (Croall and DeMartino, 1991; Saido et al., 1994; Suzuki et al., 1995). A more detailed understanding and elucidation of the biochemical events underlying calpain activation in FMCM may prove useful, since selective calpain inhibitors can be developed and screened for neuroprotection potential.

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