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Calpain cleaves methionine aminopeptidase-2 in a rat model of ischemia/reperfusion

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ABSTRACT

Ischemic stroke results in multiple injurious signals within a cell including dysregulation of calcium homeostasis. Consequently, there is an increase in the enzymatic activity of the calpains, calcium dependent proteases that are thought to contribute to neuronal injury. In addition, cellular stress due to ischemia/reperfusion also triggers a decrease in protein translation through activation of the unfolded protein response (UPR). In the present study we found that methionine aminopeptidase 2 (MetAP2), a critical component of the translation initiation complex, is a calpain substrate. *In vitro* calpain assays demonstrated that while MetAP2 has autoproteolytic activity, calpain also produces a stable proteolytic fragment at 50 kDa using recombinant MetAP2. This 50 kDa fragment, in addition to a 57 kDa fragment was present in *in vitro* digestions of rat brain homogenates. Production of these fragments was inhibited by calpastatin, the endogenous and specific inhibitor of calpain. Using an *in vivo* middle cerebral artery occlusion (MCAO) model only the 57 kDa fragment of MetAP2 was observed. These data suggest that calpain activation in stroke may regulate MetAP2-mediated protein translation giving calpains a larger role in the cellular stress response than previously determined.

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Abbreviations: ATF4, activating transcription factor 4; CAST, calpastatin; CHOP, C/EBP homologous protein; CPN, calpain; eif, eukaryotic initiation factor; MCAO, middle cerebral artery occlusion; MetAP2, methionine aminopeptidase 2; PERK, PKR-like endoplasmic reticulum kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UPR, unfolded protein response

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1. Introduction

Stroke is one of the leading causes of disability in the USA affecting over 7 million people and ischemic stroke accounts for 87% of that total (Roger et al., 2011). Ischemic stroke is caused by a decrease in blood flow to a localized area of the brain preventing blood-borne oxygen and glucose from reaching the cells within the area. This creates a focal area of necrosis called the core that continues to grow over the days and weeks following a stroke (Dirnagl et al., 1999). The penumbra surrounds the core, and consists of an area of cells undergoing progressive apoptosis, autophagy, and necroptosis (Degterev et al., 2005; Edinger and Thompson, 2004; Xu and Zhang, 2011) that likely involves the activity of the calpain protease.

Calpains are ubiquitously expressed calcium activated endoproteases. The two major isoforms of calpain found in brain are known as calpain I and calpain II, both isoforms are active at neutral pH. These isoforms are also active in the brain following stroke, and calpain activity increases over the hours following the ischemic event. For example, calpains have been shown to selectively proteolyze structural, signaling, and calcium-regulating proteins (Goll et al., 2003; Sun et al., 2008) such as spectrin, MAP-2, and in caspase 3 models of brain ischemia (Bevers and Neumar, 2008; Hong et al., 1994; Pettigrew et al., 1996; Sun et al., 2008). Direct evidence of calpain's activation in stroke comes from work utilizing the formation of stable spectrin break-down products (SBDPs) observed at 150–155 kDa. Analysis of SBDPs in ischemia show a small to moderate initial increase within the first minutes, with another wave of calpain-mediated proteolysis observed in the peri-infarct zone between 3 and 24 h after reperfusion (Hong et al., 1994; Sun et al., 2010). Other supportive evidence for calpain's role in stroke includes data showing that calpain I truncates apoptosis-inducing factor (AIF) (Cao et al., 2007) resulting in AIF translocation to the nucleus (Susin et al., 1999), an important step in some forms of programmed cell death signaling.

Additionally, cells respond to injury such as ischemia and the accompanying reperfusion by initiating the unfolded protein response (UPR) (DeGracia et al., 2002; Hu et al., 2000; Kumar et al., 2003). The UPR functions to decrease the load of misfolded or otherwise defective proteins in the stressed cell by decreasing translation of normal physiologic proteins while increasing the number of protein folding enzymes and chaperones in the endoplasmic reticulum (Harding et al., 2000; Kumar et al., 2003). Though the UPR is a pro-survival response in transient activation, chronic activation of this mechanism causes an increase in apoptosis as production of pro-apoptotic proteins such as ATF4 (activating transcription factor 4) and its downstream product C/EBP homologous protein (CHOP) increase (Marciniak et al., 2004). Following ischemia and reperfusion, the eukaryotic initiation factor 2 (eIF2α) kinase, PKR-like endoplasmic reticulum kinase (PERK) arm of the UPR is activated(Owen et al., 2005) in which MetAP2 plays an important role. In this pathway, MetAP2 dissociation from eIF2α during the UPR results in a marked decrease in normal protein translation (Datta et al., 2001). Thus, direct cleavage of MetAP2 by calpain would be expected to contribute to altered levels of protein translation following stroke by potentially altering MetAP2/eIF2α complex formation. Cleavage of eIF2γ, the eIF2 subunit responsible for strongly binding MetAP2 to the eIF2 complex, and for orienting Metap2 to bind the eIF2α subunit (Ghosh et al., 2006; Ray et al., 1993), could also alter protein translation levels.

While calpains are established as an important contributing factor to cell death, studies suggest that such a role is complex. Research strongly suggests intimate interactions between the calpains and the caspases in initiating and propagating necrosis and programmed cell death (Neumar et al., 2003; Sun et al., 2008). Such control appears due to the fact that these enzymes not only share some of the same substrates but can directly proteolyze each other or important regulators of activity. Thus, establishing the full array of potential substrates for these enzymes will increase our understanding of the molecular mechanisms involved in controlling a cells death trajectory.

In the current study, we observed that MetAP2 is a calpain substrate *in vitro*. As typically observed, calpain proteolysis of MetAP2 resulted in the creation of a stable breakdown product that was identical in size to a fragment observed *in vivo* using a MCAO model of ischemia suggesting a role for calpain. We postulate that such a fragment may retain important biological activity and significance based upon its size and cleavage location in comparison to other described fragments of MetAP2 (Datta et al., 2007).

2. Results

2.1. *MetAP2 is an in vitro substrate of both calpain 1 and 2*

Recombinant human MetAP2 (full length MetAP2 is 478 amino acids (Datta, 2000)) was digested in the presence or absence of calpain 2 (CPN2) and immunoblotted (Fig. 1). The blot shows a novel MetAP2 cleavage fragment at 50 kDa (Fig. 1A), that does not appear in control reactions in the absence of calpain. The fragmentation pattern of calpain 1 digested MetAP2 was identical to that of MetAP2 digestion by calpain 2 (CPN1, Fig. 1B). The cleavage pattern of MetAP2 in the absence of calpain I was identical to that observed in the absence of calpain II (data not shown). Note that similar to results of Datta et al. (2007), MetAP2 produces at least two stable autoproteolytic fragments clearly shown in the absence of calpain 2 (–CPN2 panel—Fig. 1A).

2.2. *MetAP2 is an in vitro substrate of calpain 2 in brain homogenate*

To confirm calpain-mediated proteolysis of MetAP2 we performed a series of experiments using mouse brain homogenate. Homogenate was digested either in the presence or absence of calpain 2 (CPN2) and separated by SDS-PAGE. Proteolysis of MetAP2 was not observed in the absence of calpain (see Fig. 2) suggesting that MetAP2 autoproteolysis, observed in the *in vitro* digestion (Fig. 1A,—CPN2) is less active in the presence of additional factors found in the cellular milieu present in this heterogeneous preparation.

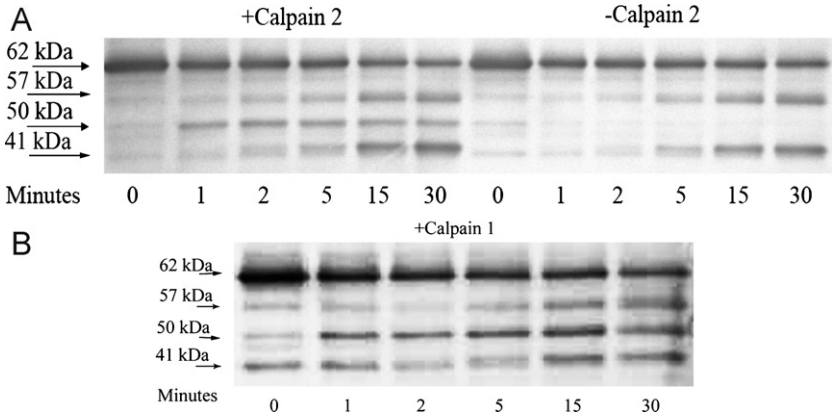

Fig. 1 – Recombinant MetAP2 protein is an *in vitro* substrate of calpain. (A) Representative Western Blot showing digestion of MetAP2 by calpain 2 over time. Several stable fragments are produced in both the presence (+) or absence (−) of calpain 2. However, a product at 50 kDa is produced in the presence of calpain only. (B) Incubation with calpain 1 shows a similar pattern of MetAP2 proteolysis and produces an identical 50 kDa fragment as that of calpain 2. Arrows indicate migration of molecular weight markers.

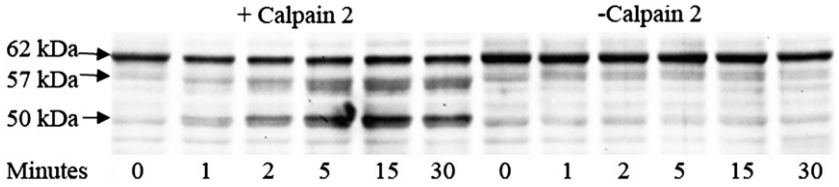

Fig. 2 – MetAP2 is an in vitro substrate of calpain 2 in mouse brain homogenates. A representative Western blot showing that calpain 2 produces proteolytic products at both 50 and 57 kDa of MetAP2 in mouse brain homogenate. Arrows indicate migration of molecular weight markers.

For example, based on the current understanding of MetAP2 interactions, it is possible that the homogenate-derived MetAP2 could be bound to an eIF2 subunit leading to inhibition of autoproteolysis. In the presence of calpain 2, two products were observed. One product was of identical molecular weight to that observed *in vitro* at 50 kDa, the other was a larger 57 kDa fragment. These data suggest that significant differences exist between digestions carried out in the presence of the cellular milieu versus a purely purified protein approach.

2.3. *MetAP2 is cleaved in a rat middle cerebral arterial occlusion (MCAO) stroke model*

Brain homogenates prepared from the rat MCAO ischemia model were analyzed to determine the similarity of the calpain-generated MetAP2 fragment pattern in this model to that observed *in vitro*. There were no significant calpain-mediated MetAP2 cleavage products observed at 1 h post-reperfusion (Fig. 3A), in contrast, MetAP2 cleavage products were present at significantly increased levels 24 h post-ischemia (Fig. 3B). There was a significant difference in the 57 kDa MetAP2 fragment of the ipsilateral as opposed to the contralateral portion of the MCAO brain (Lanes 1–8 of Fig. 3B, also C). We verified that the band at 57 kDa was most likely to be the result of calpain-mediated proteolysis of MetAP2, rather than MetAP2 autoproteolysis, by digesting contralateral homogenate in the presence or absence of calpain 2 (lanes 9 and 10 of Fig. 3B, ±CPN). In comparing the +CPN lane to the ipsilateral samples (lanes, 2,4,6,8) we noted that the fragmentation pattern was duplicated suggesting that the observed band at approximately 57 kDa is likely due to calpain activity.

2.4. *Calpastatin inhibits proteolysis of MetAP2 in vitro*

To further support a role for calpain-mediated cleavage of the 57 kDa MetAP2 fragment observed in the rat MCAO model, contralateral MCAO homogenate was digested in the presence of calpain in the presence of the endogenous and specific calpain inhibitor calpastatin (CAST) (Croall and DeMartino, 1991; Goll et al., 2003). Neither the 50 kDa nor the 57 kDa fragments were observed in the presence of calpain plus CAST, contrasting sharply with cleavage patterns seen in both the *in vitro* and *in vivo* digestions (compare Fig. 3D with Figs 1A and 2).

3. Discussion

Following a stroke, cell death occurs quickly in the core region, progressing into the penumbral area over a period of days and even weeks (Dirnagl et al., 1999). The resulting disruption in calcium signaling has been shown to activate calpains (Neumar et al., 2001) though the full array of calpain substrates have not been fully investigated. In addition,

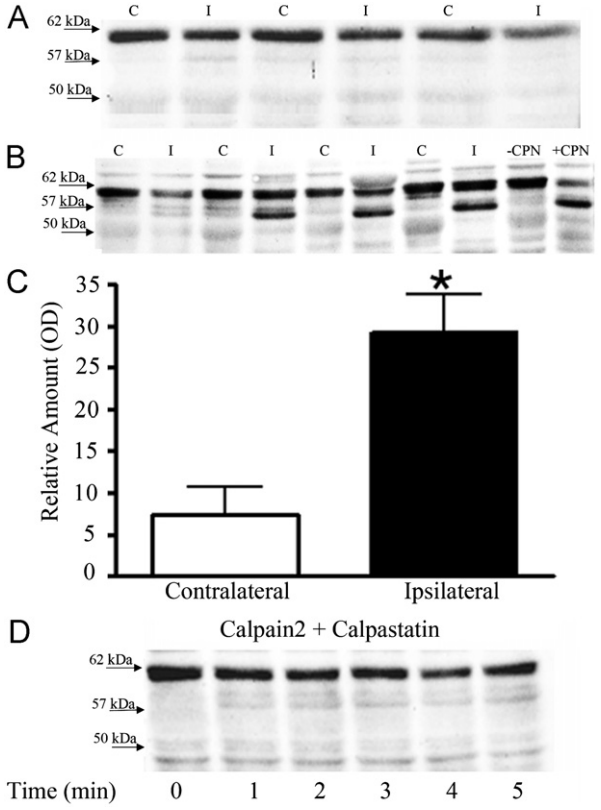

Fig. 3 – MetAP2 is cleaved by calpain in a rat middle cerebral arterial occlusion (MCAO) stroke model. (A) Tissue harvested at 1 h post-reperfusion did not show a difference in the formation of a proteolytic fragment between contralateral (C) and ipsilateral (I) brain. (B) Western blotting of MCAO brain tissue harvested 24 h post-ischemia is shown in lanes 1–8. Lanes 9 and 10 show the *in vitro* digestion of contralateral MCAO brain sample in the presence (+CPN) or absence (–CPN) of calpain 2. A 57 kDa fragment is clearly observed in 3 of 4 samples (I) that runs at an identical molecular weight on SDS-PAGE as calpain-treated brain homogenates (+CPN). (C) A significant increase in the novel 57 kDa calpain-mediated MetAP2 cleavage product was clear at 24 h post-ischemia (*=p=0.02 by Students t-test, two-tailed, paired, n=4). (D) Treatment of contralateral MCAO brain samples in the presence of calpain and calpastatin indicates that the 57 kDa fragment produced by calpain 2 is blocked by the only known endogenous calpain inhibitor, calpastatin.

cellular stress due to ischemia and reperfusion activates the PERK arm of the UPR (DeGracia et al., 2002) including dissociation of a critical ribosomal component, MetAP2, from phosphorylated eIF2α (Boyce et al., 2005; Brush et al., 2003; Datta et al., 2001; Harding et al., 2000). The present study sought to determine the interaction between the calpain proteolytic system and the UPR through selective cleavage of MetAP2.

MetAP2 is an important contributor in the execution and regulation of the UPR. Dissociation of MetAP2 from phosphorylated eIF2α causes normal protein translation to decrease while production of pro-apoptotic proteins, such as ATF4, increase due to the presence of an internal ribosome entry site, allowing bypass scanning (Boyce et al., 2005; Dever et al., 1995; Schroder and Kaufman, 2005). Bypass scanning does not occur when MetAP2 is bound to eIF2α (Datta et al., 2006) and thus dissociation of MetAP2 from eIF2α, possibly through calpain proteolysis as observed in the current study, could lead to an increase in bypass scanning of pro-apoptotic proteins.

In the present study we investigated MetAP2 proteolysis in a rat MCAO model, consisting of a 1 h focal ischemia followed by harvest of tissue at either 1 h or 24 h post-reperfusion. While tissue at 1 h post-reperfusion did not show significant differences between contralateral and ipsilateral hemispheres of the brain, ipsilateral stroke brain tissue harvested 24 h post-reperfusion showed a marked increase in MetAP2 cleavage compared to contralateral. Note that calpain 2 activity in a model of transient global ischemia, as determined by spectrin breakdown products (SBDP)'s, is present at 1 h and peaks between 24 and 48 h (Zhang et al., 2002). The increase in levels of MetAP2 at 24 h following reperfusion, and the peaking of calpain (as indicated by SBDP's in Zhang et al. (2002)) at this time, suggests that calpain proteolysis is the cause of increased MetAP2 fragment detection. It is of note that the addition of calpastatin, the endogenous calpain inhibitor (Goll et al., 2003), caused a marked decrease in production of calpain-proteolyzed MetAP2 fragments (see Fig. 3D, compare with lanes 1 through 6 of Fig. 2).

Calpain-cleaved MetAP2 could retain some of its biological activity. MetAP2 has been shown to bind strongly to eIF2γ within amino acids 340–430 of MetAP2 (Ghosh et al., 2006). Because the polyclonal antibody used to detect MetAP2 cleavage patterns includes this domain, it seems likely that calpain-cleaved MetAP2 may retain its ability to bind eIF2 subunits. This would suggest, like other calpain substrates, that the MetAP2 fragments may have a modified activity. For example, calcineurin cleavage by calpain results in the creation of a co-factor independent calcineurin linked to a pathological state (Mohmmad Abdul et al., 2011; Rosenkranz et al., 2012). Such a retention of some MetAP2 function due to preservation of eIF2 subunit binding could be hypothesized to modulate eIF2α phosphorylation. Regulation of eIF2α phosphorylation at serine 51 is activated through the PERK arm of the UPR, inhibiting translation (Harding et al., 1999). Thus, protection of eIF2α by a calpain-generated fragment has the potential to interfere with production of pro-apoptotic proteins such as CHOP and ATF4. Additionally, if a fragment possessed the ability to keep eIF2α from becoming phosphorylated at serine 51, chronic activation of the PERK arm of the UPR may not occur. Future studies to assay the extent to which these fragments retain their physiological association are needed to verify these hypotheses.

The current study also suggests that calpain-mediated cleavage is regulated by the microenvironment with important consequences for the physiological and pathological study of calpains. We found that the addition of calpain to purified MetAP2 *in vitro* produces only a 50 kDa fragment, while both a 57 kDa and 41 kDa fragment appear to be due to MetAP2 autoproteolysis (see Fig. 1). In comparison, the ~57 kDa band seen in the MCAO model closely matches the results of the mouse brain homogenate experiment, in which

both a 50 kDa and 57 kDa fragment are produced that are clearly not present in the absence of calpain (see Fig. 3B). Therefore, we suggest that the cellular milieu or other post-translational modification that occurs *in vivo* alters the calpain cleavage site in both the MCAO *in vivo* experiment and the *in vitro* mouse brain homogenate digestion. Such an observation has precedent in the literature in which lipid membranes have been shown to alter calpain substrate specificity (Goll et al., 1992; Greenwood et al., 1993; Johnson et al., 1991; Sprague et al., 2008; Zalewska et al., 2004). For example, it is possible that MetAP2 in brain homogenate exhibits altered sites for calpain cleavage due to binding to the eIF2 subunits (or other unknown factors). While SDS-Page conditions disrupt protein interactions, the controlled digestion of MetAP2 by calpain is carried out using native proteins. Therefore, the binding of factors to MetAP2 during the digestion have the potential to influence the fragment produced.

We have shown here that MetAP2 is a substrate of calpain *in vitro,* producing a novel 50 kDa and 57 kDa fragment. MetAP2 is a substrate of calpain in an *in vivo* MCAO rat model as well, producing the novel 57 kDa fragment in 24 h post-ischemia brain tissue. The proteolysis of MetAP2 by calpain could cause a subsequent drop in protein translation and an increase in production of pro-apoptotic proteins through acceleration or simply continued maintenance of the UPR. This may play a role in the growth of the core as cells would continue to undergo degenerative processing in the penumbral tissue following injury.

4. Experimental procedure

The University of Kentucky IACUC approved all experimental procedures undertaken in this work.

4.1. *Chemicals*

Unless otherwise stated, all chemicals were from SIGMA (St. Louis, MO).

4.2. *Brain homogenate preparation*

Homogenate was prepared using commercially available mouse brain (MS-T004, from Rockland Immunochemicals, Gilbertsville, PA) in a 1× phosphate buffered saline buffer containing protease inhibitors added immediately prior to use, consisting of aprotinin (1 μg/mL), pepstatin (1 μg/mL), and phenylmethylsulfonyl fluoride at a final concentration of 1 mM. Rat brain homogenate from either ipsilateral or contralateral brain hemispheres was similarly prepared. Tissue homogenates were generated by 14 manual strokes with a dounce homogenizer, followed by centrifugation at 14,000 × *g* for 15 min at 4 °C. The supernatant was collected and protein concentration determined using the bicinchoninic acid protein assay from Pierce (Rockford, IL).

4.3. *Calpain proteolysis protocol*

In vitro calpain digestions were carried out using porcine calpain from Calbiochem (La Jolla, CA) (20 units/mL using bodipy casein as standard), with recombinant MetAP2 from R&D Systems (Minneapolis, MN). Tissue homogenate was digested with higher levels of calpain (200 units/mL) in a buffer containing 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH=7.4), 2 mM dithiothreitol (DTT), 0.1% (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate), and 2 mM $CaCl_2$. Reactions were initiated by the addition of calcium. Aliquots were removed at the indicated time points. Each aliquot was then mixed with an equal volume of 2× Laemmli buffer (250 mM Tris, pH=6.8, 20% glycerol, 12.5 mM ethylenediaminetetraacetic acid, 12.5 mM ethylene glycol tetraacetic acid (EGTA), 5% sodium dodecyl sulfate (SDS), 250 mM DTT, and 0.4% bromophenol blue). Samples were then heated to 90 °C for 5 min and samples stored at −80 °C until use. The final concentration of calpastatin added to the homogenate above (in digestions involving calpastatin) was 100 nM.

4.4. *Rat model of experimental stroke*

The MCAO rat model procedure is as previously described (Pandya et al., 2011). Briefly, rats underwent MCAO by suture-occlusion for 1 h. After 24 h of post-ischemic reperfusion, each animal was re-anesthetized by intraparietal injection of chloral hydrate and was perfused transcardially with India ink. The brain was removed *en bloc*, cut into 2-mm sections, and frozen at −80 °C (Pandya et al., 2011). The contralateral side of the brain served as the control. Frozen tissue samples were homogenized as in mouse brain homogenate preparation above.

4.5. *SDS-PAGE protocol*

Samples were separated using 10% SDS-PAGE. Following separation, proteins were transferred to a nitrocellulose membrane and blocked for 1 h in 3% bovine serum albumin (BSA) in Tris-Buffered Saline with Tween-20 (50 mM Tris–HCl, pH=7.4, 150 mM NaCl, 0.5% Tween-20; TBST). The primary antibody (SC-67234, MetAP2 (H300),Santa Cruz, CA) was diluted in 3% BSA-TBST and the blot was incubated at 4 °C overnight. An appropriate HRP-conjugated secondary antibody was added and incubated for 1–2 h at room temperature. Blots were extensively washed in TBST and developed using enhanced chemiluminescence (Pierce, Rockford, Illinois). Blots were analyzed using Bio-Rad Chemidoc XRS and Quantity One software.

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