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Selective Release of Calpain Produced αII-Spectrin (α-Fodrin) Breakdown Products by Acute Neuronal Cell Death

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Activation of calpain results in the breakdown of α II spectrin (α-fodrin), a neuronal cytoskeleton protein, which has previously been detected in various *in vitro* and *in vivo* neuronal injury models. In this study, a 150 kDa spectrin breakdown product (SBDP150) was found to be released into the cell-conditioned media from SH-SY5Y cells treated with the calcium channel opener maitotoxin (MTX). SBDP150 release can be readily quantified on immunoblot using an SBDP150-specific polyclonal antibody. Increase of SBDP150 also correlated with cell death in a time-dependent manner. MDL28170, a selective calpain inhibitor, was the only protease inhibitor tested that significantly reduced MTX-induced SBDP150 release. The cell-conditioned media of cerebellar granule neurons challenged with excitotoxins (NMDA and kainate) also exhibited a significant increase of SBDP150 that was attenuated by pretreatment with an NMDA receptor antagonist, *R*(–)-3-(2-carbopiperazine-4-yl)-propyl-1-phosphonic acid (CPP), and MDL28170. In addition, hypoxic/hypoglycemic challenge of cerebrocortical cultures also resulted in SBDP150 liberation into the media. These results support the theory that an antibody-based detection of SBDP150 in the cell-conditioned media can be utilized to quantify injury to neural cells. Furthermore, SBDP150 may potentially be used as a surrogate biomarker for acute neuronal injury in clinical settings.

Key words: Apoptosis / Biomarker / Calpain / Cell death / Excitotoxin / Fodrin / Oncosis.

Introduction

Calpain represents a class of cytosolic cysteine proteases which are activated by elevated intracellular calcium concentrations (Suzuki *et al.*, 1995; Yuen and Wang 1998). Currently, there are two major isoforms of calpain found in the central nervous system, μ-calpain and m-calpain. μ-Calpain requires micromolar quantities of calcium to be activated while m-calpain requires millimolar levels. Calcium serves as a trigger for cellular injury and calpain represents a crucial mediator of the degenerative process. Uncontrolled activation of calpain leads to cytoskeleton protein spectrin (fodrin) breakdown, degradation of many receptor proteins (EGF receptor) and enzyme systems (protein kinase C and calmodulin-dependent kinases) (Wang *et al.*, 1989) and consequently cell death (Wang *et al.*, 1996; Kampfl *et al.*, 1997). Increased proteolysis of brain spectrin has been identified as an early neurodegeneration marker following glutamate excitotoxicity, denervation, hypoxia, ischemia and apoptosis (Sihag and Cataldo, 1996). Therefore it is a good marker to track apoptosis and necrosis in injury and other neurodegenerative processes.

Maitotoxin (MTX) is a very potent marine toxin found in the dinoflagellate *Gambierdiscus toxicus*, which is eaten by the surgeon fish *Ctenochateus striatus* in which the toxin accumulates (Gusovsky and Day, 1990). MTX is known for its ability to stimulate calcium influx in excitable and non-excitable cells by both voltage-dependent calcium channels as well as receptor-operated calcium (Gusovsky and Day, 1990). We have previously demonstrated that MTX is an excellent activator of cellular calpain (Wang *et al.*, 1996a; Zhao *et al.*, 1999). It is well established that when cellular calpain is over-activated, its substrate protein, α-spectrin, is degraded into two proteolytic fragments (150 kDa and 145 kDa; Siman *et al.*, 1984; Saido *et al.*, 1992; Wang *et al.*, 1996a) called αII-spectrin breakdown products, SBDP150 and SBDP145. This distinct pattern of fragmentation of α-spectrin has been used as an assay for calpain activity in intact cells (Bartus *et al.*, 1995; Hajimohammadreza *et al.*, 1995; Wang *et al.*, 1996; Bahr *et al.*, 1997). A specific anti-SBDP150 antibody recognizes just the SBDP150 in injured cells undergoing oncosis or apoptosis (Wang, 2000).

In this study we show that calpain-generated α-spectrin breakdown products, including SBDP150, are selectively released into cell-conditioned medium from MTX-treated human neuroblastoma SH-SY5Y cells. Using SBDP150 release as a biomarker, we quantified injury of cerebellar granule neurons caused by excitotoxins and its inhibition by calpain inhibitors. Similarly, we also quantified the hypoxic/hypoglycemic injury in primary neuronal cultures.

Results

SBDP150 Release into Cell-Conditioned Medium Resulting from Maitotoxin-Induced SH-SY5Y Cell Death

We previously demonstrated that treatment with the calcium channel opener maitotoxin induces SH-SY5Y neuroblastoma cell death (Wang *et al.*, 1996). Maitotoxin treatment is indeed associated with a biphasic membrane blebbing followed by bleb dilation and oncotic cell death (Nath *et al.*, 1996). We also established that maitotoxin (MTX) induced calpain-mediated αII-spectrin breakdown in SH-SY5Y cells between concentrations of 0.1 nM to 30 nM with the peak at 1 nM (Wang *et al.*, 1996). Cell pellets from untreated cells showed intact αII-spectrin (280 kDa), while MTX (0.3 nM) treated cells have αII-spectrin partially degraded into its major breakdown products of 150 kDa (SBDP150) and 145 kDa (SBDP145) (Figure 1A). We have developed an SBDP150-specific antibody based on the neo-N-terminus of SBDP (NH2-GMMPR; Saido *et al.*, 1992). Indeed, anti-SBDP150 antibody selectively detected SBDP150 in MTX-treated cells (Figure 1B).

To confirm that SBDP150 was generated by calpain, control SY5Y cell lysate was digested with 3 µg of purified µ-calpain for 5 – 90 min (see Materials and Methods). The Western blot (Figure 2A) shows that SBDP150 increased with time until it peaked at about 30 minutes, after which it attenuated (Figure 2B). This may be due to the additional breakdown into other smaller SBDPs (Yokota *et al.*, 1995). Intact αII-spectrin was not detected with this antibody.

We speculate that some of the SBDPs might find their way into the SH-SY5Y cell-conditioned medium after the initial cell injury by MTX. Cell-conditioned medium was therefore collected 90 min after MTX-induced injury and the samples were examined for the presence of spectrin signals by Western blots. Only a small amount of intact αII-spectrin (280 kDa) was observed in the cell-conditioned media from 0.1 nM MTX-treated cells (Figure 3A and C). Yet both SBDP150 and SBDP145 were readily observed in a cultured medium conditioned with cells treated with various concentrations of MTX (0.1, 0.3 and 1 nM; see Figure 2A). Using anti-SBDP150, we also observed the robust appearance of SBDP150 bands with all MTX-treated cells (Figure 3B). In striking contrast, no

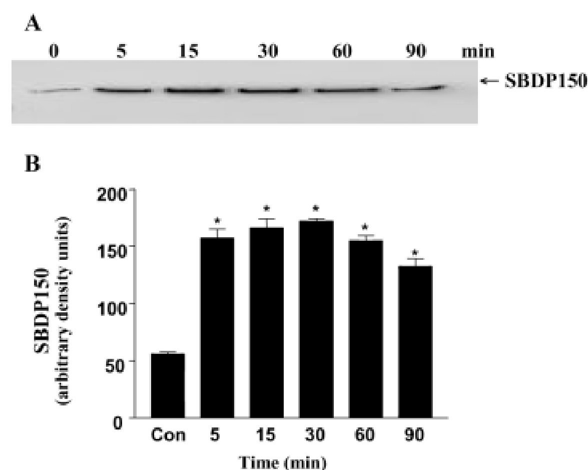

Fig. 2 *In vitro* Digestion of α-Spectrin in Control Cell Lysate with µ-Calpain.
(A) Western blot of SH-SY5Y cell lysates digested with Calpain. (B) Quantification of SBDP150, which peaked around 30 minutes after which it attenuated. Data are mean values ± SEM (bars); n=3. Values different from control are indicated: *p <0.001 by ANOVA test.

SBDP150 was detected in the control cell medium. The SBDP150 signal can be readily quantified densitometrically (Figure 2C).

SBDP150 Release Is Time-Dependent and Parallels Cell Death

SH-SY5Y cells were challenged with 0.1 nM MTX (except control) in a time course experiment (0 – 16 h). The immunoblot shows an increase of SBDP150 release in a time-dependent manner (Figure 4A). As early as 1 h there is a significant release of SBDP150, which continued to increase up to 16 h (Figure 4B). Cell death increased in a time course closely parallel but preceded SBDP150 release (Figure 4C).

Inhibition of SBDP150 Release into SH-SY5Y Cell-Conditioned Media by Calpain Inhibitor

Maitotoxin causes calpain activation in SH-SY5Y cells (Wang *et al.*, 1996). To determine whether SBDP150 was truly calpain mediated, cells were pre-treated for 1 h with

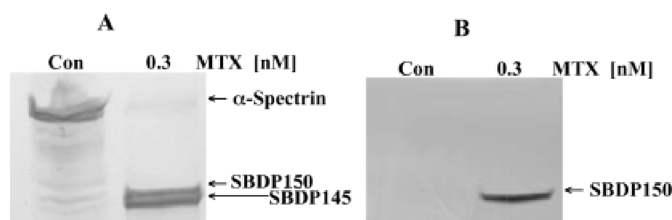

Fig. 1 Anti-Spectrin Western Blot Analysis of Cell Pellets from Control and MTX-Treated SH-SY5Y Cells.
SH-SY5Y cells were left untreated or treated with maitotoxin (MTX, 0.3 nM) for 3 h. Intact α-spectrin (left panel) or specific SBDP150 (right panel). The arrows indicate intact αII-spectrin or SBDP150 and SBDP145. Data are representative of three separate experiments.

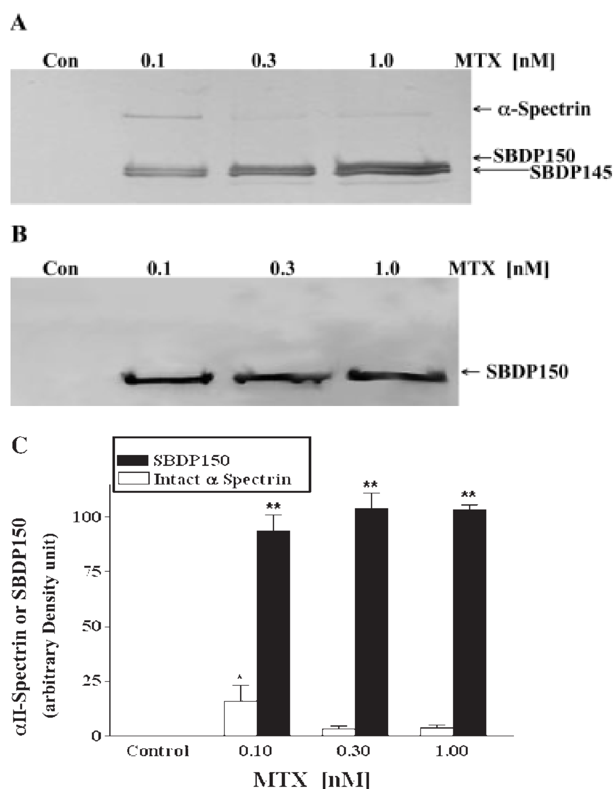

Fig. 3 Western Blot and Quantification of Intact α-Spectrin and Specific SBDP150 Release in Cell-Conditioned Medium.
SH-SY5Y cells were treated with MTX (0.1-1 nM) for 3 h. Cell-conditioned medium was collected and analyzed by Western blots probed with (A) anti-αII-spectrin antibody or (B) specific anti-SBDP150 antibody (bottom panel). With increase of injury, (C) densitometric analysis of the release of intact α-spectrin based on anti-α-spectrin (open bar) and SBDP150 based on anti-SBDP150 (closed bar). Data are mean values ± SEM (bars); n=3. Data significantly different from control are indicated: *$p<0.05$ and **$p<0.001$ by ANOVA test.

various protease inhibitors. Carbobenzoxy-valyl-phenylalaninal (MDL28170) was used as a selective calpain inhibitor, carbobenzoxy-Asp-$CH_2OC(O)$-2,6-dichlorobenzene (Z-D-DCB) as a caspase inhibitor, Z-Ile-Glu(OtBu)-Ala-Leu-CHO (IGAL) and lactacystin as proteasome inhibitors, CA074Me as a cathepsin B inhibitor and (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucyl-amido-3-methylbutane ethyl ester (E64d) as a lysosomal cysteine protease inhibitor. Western blots were performed to examine the effectiveness of these compounds on SBDP150 release (Figure 5A). The calpain inhibitor, MDL28170 (Figure 5B),

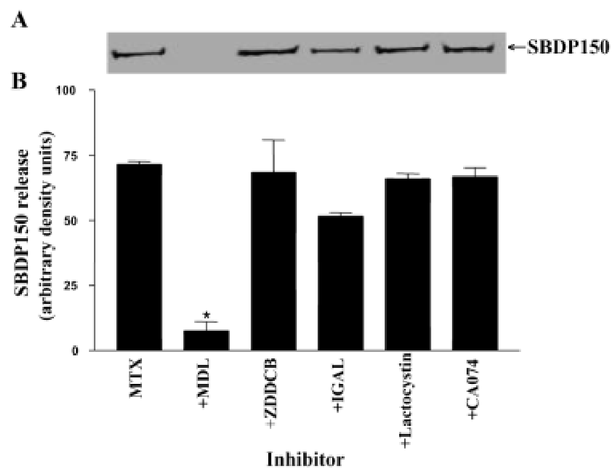

Fig. 5 Effect of Protease Inhibitors on SBDP150 Release.
SH-SY5Y cells were pre-treated with various protease inhibitors. (A) Western blotting was performed with 20 µg of protein to check the effects of the compounds. (B) Quantification of Western blot: calpain inhibitor, MDL28170, was the only inhibitor that significantly (*$p<0.001$ by ANOVA test) reduced SBDP150 release more than control MTX (0.3 nM) treated cells. Results are mean ± SEM.

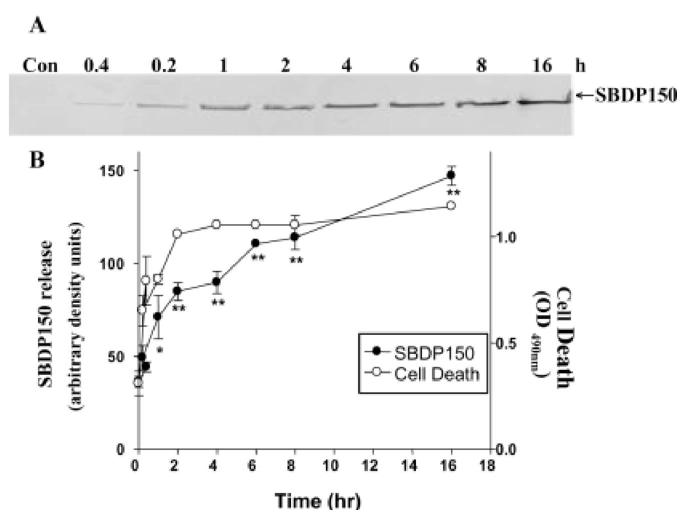

Fig. 4 Time Course of SBDP150 Release in Comparison to Cell Death.
SH-SY5Y cells were challenged with 0.1 nM MTX (except control). (A) Western blot of the time course (15 min–16 h) with anti α-SBDP150. (B) Densitometric quantification and analysis of the blot shows time-dependent increase in SBDP150. Data are mean values ± SEM (bars) and n=3. Data significantly different from control are indicated: *$p<0.05$ and **$p<0.01$ by ANOVA test. Cell death was monitored using a cytotoxicity assay. (C) Cell death (○) is seen to be parallel to SBDP150 release (●).

was the only inhibitor tested that provided significant protection from breakdown and reduced SBDP150 release from control MTX (0.3 nM) treated cells. This validates that SBDP150 release is an event that is mediated by calpain.

SBDP150 Release from Excitotoxin-Treated Cerebellar Granule Neurons

Rat cerebellar granule neurons were challenged for 20 h with different concentrations of the glutamate receptor agonists N-methyl-D-aspartate (NMDA) and kainate. Some cells were pre-incubated for one h with D-CPP, *R*(–)-3-(2-carbopiperazine-4-yl)-propyl-1-phosphonic acid (CPP; an NMDA receptor antagonist) and calpain inhibitor, MDL28170, respectively, at the highest dose of NMDA or kainate (400 μM) (Figure 6A). It has been well documented that excitotoxins such as NMDA and kainate induce excessive Ca^{2+} influx and sustained intracellular Ca^{2+} elevation leading to cell death. In our model, we previously demonstrated that the granule neurons die from mixed oncotic/apoptotic cells (Nath *et al.*, 1998). Twenty-five μM of NMDA- or kainite-treated cells served as controls, as they do not evoke excitotoxic or SBDP150 release responses. We observed a dose-dependent increase of SBDP150 with kainate treatment (beginning at 50 μM) (Figure 6). For NMDA, only the highest concentration used (400 μM) produced significant and marked SBDP150 release (Figure 6). There was a significant and marked inhibition of SBDP150 release with NMDA when cells were pretreated with the NMDA receptor-specific antagonist, CPP, which suppresses NMDA receptor-mediated calcium influx. Pretreatment with the calpain inhibitor MDL28170 also inhibited SBDP150 release, not by glutamate channel inhibition but rather by downstream calpain activation. In kainate-treated cells, significant attenuation of SBDP150 release occurred with MDL 28170 but not with CPP.

SBDP150 Release from Oxygen- and Glucose (OGD)-Deprived Cerebrocortical Cultures

Rat cerebrocortical mixed cultures were brought to hypoxic and hypoglycemic conditions as previously established (Nath, 1998). In this model, we previously demonstrated that only the neurons selectively vulnerable to combined hypoxia and hypoglycemia, which causes energy depletion which in turn leads to intracellular calcium elevation, exhibited mixed apoptotic and oncotic cell death (Nath *et al.*, 1998). The normal glucose level (22 mM) was reduced to 1.85, 1.6, 1.35 and 1.1 mM and oxygen deprivation (1% O2) was sustained for 2 h. The cells were then reconstituted in regular glucose and oxygen for 24 h (see Materials and Methods). We observed that in the presence of oxygen deprivation, there was a correlation between the decreasing glucose levels and the increase in SBDP150 release during the 24 hours post injury. At the lowest glucose level used (1.1 mM), SBDP150 release was particularly significant. SBDP150 also correlated well with injury in primary cultures exposed to hypoxic and hypoglycemic conditions (based on microscopic inspection). SBDP150 release was a time-dependent process, as there was very little SBDP150 release if cell-conditioned medium was sampled immediately after the 2 h oxygen and glucose deprivation (Figure 7A, B). We also observed increased injury, as well as increased SBDP150 release, 24 hours post 90 minutes of oxygen and glucose deprivation (OGD), but to a lesser extent when compared to those with 2 h of deprivation (data not shown).

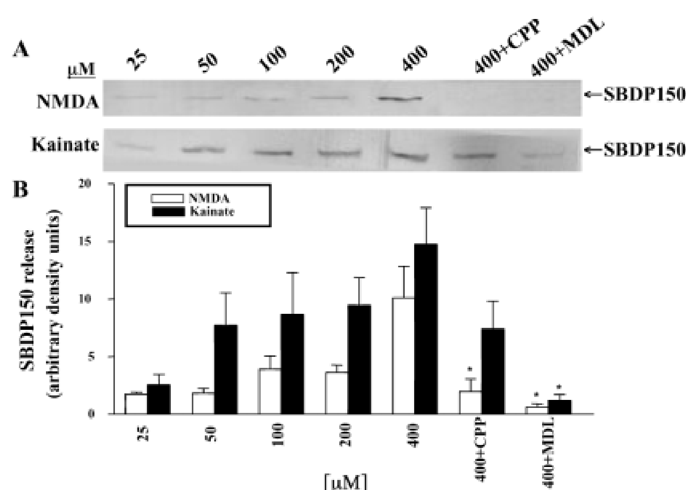

Fig. 6 SBDP150 Release from Excitotoxin-Treated Cerebellar Granule Neurons.
Cells were challenged for 20 h with various concentrations of NMDA (□) and kainate (■). Cells were pre-incubated for an hour with CPP (an NMDA antagonist) and MDL28170 where noted. (A) Western blot showing the dose-dependent increase of SBDP150 release with the excitotoxins. (B) Quantification of Western blot: the calpain inhibitor MDL 28170 significantly attenuates the excitotoxicity in both the NMDA- and kainite-treated cells whereas CPP was more effective in blocking the NMDA-induced injury pathway. Data are mean ± SEM (bars) and n=3. Data significantly different from 400 μM excitotoxin are indicated: $^*p<0.05$ by ANOVA test.

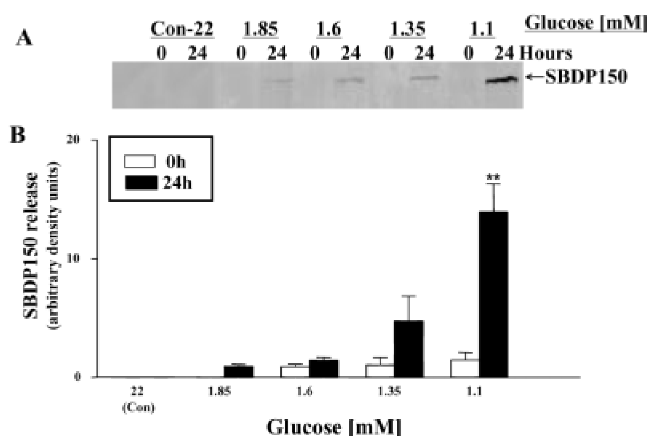

Fig. 7 SBDP150 Release from Oxygen- and Glucose-Deprived Cells.
(A) Western blot showing 0 and 24 h samples that correlate to dose-dependent hypoglycemia and 2 h hypoxia. (B) Densitometric quantification of Western blots showed a significant increase of SBDP150 (*p=0.0002 by ANOVA test) in cells that were cultured in 1.1 mM glucose, subjected to 2 h oxygen deprivation and were harvested at 24 h post-treatment. Data are mean ± SEM (bars) and n=3.

Discussion

In this report, we showed for the first time that spectrin breakdown products are seen and can be quantified from the cell-conditioned media. We illustrate that spectrin breakdown products (*e.g*. SBDP150) are released into the cell-conditioned media, from at least three different neuronal cell injury paradigms: neuroblastoma SH-SY5Y cells challenged with maitotoxin (Figures 3 and 4), rat cerebellar granule neurons subjected to excitotoxins (NMDA and kainate) (Figure 6), and rat cerebral cortical cultures subjected to oxygen and glucose deprivation (OGD; Figure 7). The time course of SBDP150 release into the media corresponded with cell death (Figure 4); thus it is potentially a useful marker for neuronal injury. Since SBDP150 is selectively produced by calpain activation (Figure 5), SBDP150 release is also an excellent marker for calpain activity in injured neurons. Calpain-generated SBDP150 can be found in SH-SY5Y in oncotic necrosis with maitotoxin challenge or from apoptosis induced by staurosporine challenge (Nath *et al.*, 1996). SBDP150 is also generated from mixed oncotic/apoptotic death when granule neurons are subjected to excitotoxin (NMDA, kainate) and cortical neurons are subjected to oxygen glucose deprivation, as was previously demonstrated (Nath *et al.*, 1998, 2000). Thus, it is likely that SBDP release can be used as a marker to monitor the extent of both necrosis and apoptosis.

Neuronal culture medium mimics intercellular fluid found in the brain. Proteins that are in intercellular fluid find their way into the cerebral spinal fluid (CSF). Since the spectrin breakdown product is found in injured neuron-conditioned medium, it is highly likely that SBDP150 is present in CSF, subsequent to ischemic or traumatic brain injury *in vivo*. Recently there has been increased interest in searching for biomarkers for both chronic neurodegeneration (such as Alzheimer's disease) as well as acute neurological disorders (such as stroke and traumatic brain injury). In fact, in the last few years Tau protein has been found to be significantly increased in the CSF of early human AD patients (Riemenschneider *et al.*, 1996; Tapiola *et al.*, 1997; Kurz *et al.*, 1998; Green *et al.*, 1999). In addition, amyloid β peptides are also increased in CSF from AD patients (Otto *et al.*, 2000; Riemenschneider *et al.*, 2000; Tapiola *et al.*, 2000). In contrast, to our knowledge S100B protein is the only extensively studied biomarker (*e.g*. detected in CSF and serum) to assess the progression of acute neurological disorders (Jonsson *et al.*, 2001; de Kruijk *et al.*, 2001). But since S100B is derived from glia cells, we could not perform a direct comparison between S100B and SBDP150 release in our cell culture paradigm. Nuclear magnetic resonance imaging (MRI) has been used with some success to monitor the state of brain metabolism after acute brain injury. It is also becoming increasingly important to use a biomarker to monitor the efficacy of therapeutic treatment for acute brain injury in clinical settings. Clearly this field is in need of a robust and sensitive biomarker. Our results in this study raised the possibility that SBDP150 and other spectrin breakdown products (SBDP) are potential biomarkers in CSF for acute brain injury. As we have now validated that SBDP is an excellent biomarker *in vitro* the natural next step is to detect SBDPs in CSF from *in vivo* animal models of ischemic and traumatic brain damage. In fact, we have recently demonstrated that SBDPs are detectable and significantly elevated in the CSF in rats after traumatic brain injury (unpublished results). Another important step is to develop a quantitative and sensitive ELISA or sandwich ELISA method to measure SBDP. In summary, our study is the first step toward a potentially important advancement in developing a biomarker for human acute brain injury.

Materials and Methods

Reagents

Carbobenzoxy-valyl-phenylalaninal (MDL 28170), also called calpain inhibitor III; carbobenzoxy-Asp-$CH_2OC(O)$-2,6-dichlorobenzene [Z-D-DCBZ-Ile-Glu(OtBu)-Ala-Leu-CHO] (PSI; IGAL), and lactacystin were purchased from Calbiochem. Cell permeable cathepsin B inhibitor CA074(Me) was purchased from Peptide International. N-methyl-D-aspartate (NMDA) and (2S,3S)-*trans*-epoxysuccinyl-L-leucyl-amido-3-methylbutane ethyl ester (E64-d) was purchased from Sigma. *R*(–)-3-(2-Carbopiperazine-4-yl)-propyl-1-phosphonic acid (D-CPP) was purchased from RBI. Anti-spectrin (fodrin) (monoclonal) antibody was obtained from Affinity-labs, UK. Anti-SBDP150 raised in rabbits against the new N-terminal of SBDP150 (H_2N-Gly-Met-Met-Pro-Arg) (polyclonal) was made at Senju pharmaceuticals (Kobe, Japan) and was affinity purified as described elsewhere (Saido *et al.*, 1992).

Maitotoxin Challenge of SH-SY5Y Neuroblastoma Cells

Human neuroblastoma SH-SY5Y cells were cultured on a 12-well plate until confluent. Cells were periodically fed with DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. All cells were washed three times in serum-free medium before experimental protocols. Cells were challenged with 0.1 nM–1 nM maitotoxin, a calcium channel activator (Nath *et al.*, 1996) for 3 hours. The supernatant or cell-conditioned media were collected for protein extraction and analysis. Time course experiments were performed on 0.1 nM maitotoxin-challenged SH-SY5Y cells between 15 min to 16 h. Twenty-five µl of the supernatant were collected after each time-point and saved for cell viability assay using the CytoTox 96 cytotoxicity kit (Promega) to measure cytosolic lactate dehydrogenase (LDH) release into the medium. The rest of the conditioned media or supernatant was collected for protein extraction and analysis. SH-SY5Y cells were pre-treated with various inhibitors for one hour before (0.3 nM) maitotoxin challenge: MDL28170 at 20 µM; Z-D-DCB at 100 µM; IGAL at 10 µM; lactacystin at 10 µM; CA074(Me) at 10 µM and E64d at 32 µM. After 3 h incubation, protection by the different inhibitors was checked by analyzing the collected supernatant.

µ-Calpain Digestion of Control SH-SY5Y Cell Lysate

SH-SY5Y cells were cultured to confluence. Supernatant was removed and lysis buffer containing 20 mM Tris HCl, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 5 mM EDTA and 1 mM DTT was added to the cells and the mixture was frozen overnight. After thawing the cells for 2 hours on ice, cell debris was removed by centrifuging for 5 min and the supernatant was collected as a cell lysate. Cell lysate (60 µg of protein) was digested at room temperature with 3 µg of µ-calpain for 5, 15, 30, 60 and 90 minutes. The reaction was stopped by the addition of SDS sample buffer, and SDS PAGE and Western blots were performed. The blots were probed with anti-SBDP150 antibody.

Oxygen-Glucose Deprivation (OGD) in Cerebral Cortical Cultures

Primary rat cell isolation was carried out in compliance with the NIH guide for animal care and use of laboratory animals and the Parke-Davis animal use committee. Cerebrocortical cells were harvested from fetal rats (Sprague-Dawley) on day 18 of gestation and cultured with Dulbecco's modified Eagle's medium/F12 (Gibco-BRL) medium containing 10% equine serum and 6% fetal bovine serum (heat-inactivated) in 24-well poly-L-lysine coated plates as described previously (Hajimohammadreza *et al.*, 1995; Nath *et al.*, 1998). Non-neuronal cell division was halted at 3 days by adding 35 µg/ml uridine and 15µg/ml 5-fluoro-2'-deoxyuridine. On day 17 post-plating, the cultures were washed three times with serum-free medium. The cultures were then challenged with hypoxy-hypoglycemia for 120 minutes with an exposure atmosphere in a gas incubator of 1% O_2, 8% CO_2 and 91% N_2 in an exposure medium of Hank's balanced salt solution containing 1.8 mM Ca^{2+}, 0.8 mM Mg^{2+} and different D-glucose concentrations (1.1, 1.35, 1.6, 1.85 mM). The plates were then reconstituted to normal serum-free medium and then placed in an oxygenated incubator (21% O_2, 8% CO_2 and 71% N_2) until 24 hours after the experiment initiation. Normooxic/normoglycemic (22 mM) cultures with the same number of medium changes were used as control. The supernatant was collected for protein analysis and Western blotting.

Excitotoxicity in Cerebellar Granule Neurons

Cerebellar granule neurons were isolated from 7 day-old rat pups (Sprague-Dawley; Charles River Laboratories) as previously described (Nath *et al.*, 1998). Briefly, the meninges were removed from the cerebellum and the minced tissues was trypsinized (0.25mg/ml) for 15 minutes at 37 °C. The trypsinized tissue was then washed in 0.4 mg/ml DNase I for 5 min. The tissue was triturated three times in 0.4 mg/ml DNase I. The combined supernatant was divided into four centrifuge tubes, and 3 ml of 4% (w/v) bovine serum albumin/1 mM $MgSO_4$ were layered underneath the supernatant. The cells were then centrifuged at 600 *g* and washed in HEPES buffered salt solution (20 mM HEPES; 0.59 mM EDTA; pH 7.3) for 5 min. The pellet was resuspended and cultured (about one cerebellum per 12-well plate) in 2 ml feeding medium (Dulbecco's modified Eagles' medium plus 30 mM KCL, 10% fetal bovine serum, 5 mg/ml insulin, and 100 U/ml penicillin/streptomycin) in a 37 °C humidified 5% CO_2 incubator. Half of the medium was replaced with fresh medium containing 20 µM arabinosidase after 24 h. More than 95% of the population of cells in these cultures were neurons based on routine inspection. At the beginning of the experiment, 7 day-old cultures were washed three times with serum-free Dulbecco's modified Eagle's medium. Some of the samples were pre-incubated with 200 µM CPP and 20 µM MDL for one hour. The cultures were then challenged with different concentrations of NMDA and kainate (25, 50, 100, 200, 400 µM) in 120 mM HEPES. Cultures were maintained for 20 h, at which time cell viability was monitored using the CytoTox 96 cytotoxicity kit (Promega) to measure LDH release into the medium. The supernatant was collected for analysis.

Protein Extraction and Protein Sample Analysis

The cells were spun down and the supernatant or the cell-conditioned medium from the cells of each experiment (unless otherwise specified) was collected and the protein was precipitated with 20% TCA. The precipitated protein was neutralized with 50 µl of 3 M Tris base. Protein samples were quantified using the Bio-Rad D_c Protein Assay (Cat.# 500–0116). Twenty µg protein were electrophoresed on sodium dodecyl sulfate-poly acrylamide gel electrophoresis (10%–20% polyacrylamide gels) (Novex) with a Tris-glycine running buffer system and then transferred to a PVDF membrane using a semi-dry electrotransferring unit (Bio-Rad) at 20 mA for 2 h. The blots were probed with anti-α-SBDP150 (rabbit polyclonal) antibody or intact α-spectrin where noted. Blots were developed with a nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Densitometric analysis of Western blots was performed using a color scanner (Umax UC603) and the NIH program Image 1.5.

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