

Activation of apoptosis-linked caspase(s) in NMDA-injured brains in neonatal rats

Rathna Nath*, Michele Scott, Ravi Nadimpalli, Rita Gupta, Kevin K.W. Wang

Laboratory of Neuro-biochemistry, Department of Neuroscience Therapeutics, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, Michigan, 48105, USA

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Abstract

Unilateral injection of 50 nmol of *N*-methyl-D-aspartate (NMDA) into the left posterior striatum of 7 day-old rat pups induces massive neuronal loss in the ipsilateral hemisphere in 5 days. In this model of excitotoxicity, the form of neuronal death (necrosis vs apoptosis) has not been clearly addressed. Here we report evidence of DNA laddering in the ipsilateral hemisphere 24 h after the NMDA injection. Activation of apoptosis-linked caspase(s) was also identified, as evidenced by (i) the formation of caspase-produced 120 kDa alpha-spectrin breakdown product (SBDP120) and (ii) increase in hydrolysis of caspase-3 substrate acetyl-DEVD-7-amido-4-methylcoumarin in the homogenate from the ipsilateral hemisphere. Lastly, we note that i.p. injection (100 mg/kg) of a pan caspase inhibitor Z-D-DCB attenuates the levels of SBDP120. Our results suggest the presence of caspase-activation in this rat pup model of NMDA toxicity. © 2000 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Caspase; ICE-like; Excitotoxicity; Neuronal death; Protease inhibitor

1. Introduction

Programmed cell death (or apoptosis) is characterized by a number of features, including cytoplasmic shrinkage, DNA cleavages in the nucleosome linker regions and chromatin condensation along the inside surface of the nuclear envelope as a result of DNA fragmentation (Cohen, 1993). Terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL) has been a popular method to identify apoptotic cells as it detects

increases of fragmented DNA (Charriaut-Marlangue et al., 1995). The mode of cell death during in vivo model of cerebral ischemia or excitotoxicity has been generally believed to be necrotic. Linnik et al. (1993) was the first group to challenge this notion by reporting evidence of DNA laddering and TUNEL labeling in a rat model of focal ischemia. Shortly after, MacManus et al. (1993) reported similar findings in a 4-vessel-occlusion global ischemia model. Since then numerous reports appeared documenting the presence of apoptosis in other focal or global ischemia or kainate toxicity models (Chen et al., 1997; Filipkowski et al., 1994; Heron et al., 1993; Li et al., 1995a; Petito et al., 1997). Besides DNA laddering and TUNEL, electron micrographic analysis has been employed in identifying apoptotic neurons. Yet using similar techniques, several research groups found little or no evidence of classic apoptosis (Dessi et al., 1993; Scott and Hegyi, 1997; van Lookeren Campagne and Gill, 1996). Using TUNEL method solely to identify apoptotic cells has now been criticized based on the fact that it only detects the increase of fragmented DNA,

Abbreviations: NMDA, *N*-methyl-D-aspartate; α -spectrin, nonerythroid alpha-spectrin; SBDP, α -spectrin breakdown product; SBDP120, 120 kDa breakdown product; Z-D-DCB, carbobenzoxy-Val-Ala-Asp(OEt)-CH₂OC(O)-2; 6-dichlorobenzene; H and E, hematoxylin and eosin; TUNEL, terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labelling; ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; ac-YVAD-AMC, acetyl-Tyr-Val-Ile-Asp-7-amido-4-methylcoumarin.

* Corresponding author. Tel.: +1-734-622-2389; fax: +1-734-622-7178.

E-mail address: rathna.nath@wl.com (R. Nath).

which occurs in both apoptosis and necrosis (Charriaut-Marlangue and Ben-Ari, 1995). Recently, neuronal apoptosis has also been identified in spinal injury and traumatic models (based on evidence of DNA laddering and apoptotic nuclei morphology). Ideally, a more definitive biochemical marker for apoptosis would be much preferred to define the presence of apoptosis.

In recent years, caspase activation has been strongly linked to apoptosis (Nicholson and Thornberry, 1997). Caspase-3 and 7 are two highly related mammalian members that mediate apoptosis. Caspase-3 (previously called CPP32, Yama, apopain) is found in all cell types, including central neurons (Ni et al., 1997). Interestingly, caspase-7 mRNA is expressed in all tissues but absent in the brain (Juan et al., 1997). Consistent with that, caspase-3 knockout mice have a major defect in the machinery of neuronal apoptosis during development, while peripheral tissues are spared (Kuida et al., 1996). Neuronal or neural cells in culture, when subjected to pro-apoptotic challenges (staurosporine, potassium or growth factor deprivation), always result in caspase-3 activation (Armstrong et al., 1997; Koh et al., 1995; Nath et al., 1996a; Ni et al., 1997; Wiesner and Dawson, 1996). During apoptosis, caspase-3 apparently cleaves various cellular proteins such as poly(ADP-ribose) polymerase (PARP) (Lazebnik et al., 1994) and nonerythroid α -spectrin (Martin et al., 1995; Nath et al., 1996a). In our previous study, we found that although both calpain and caspase-3 are capable of fragmenting α -spectrin, only caspase produces the distinct 120 kDa breakdown product (SBDP120) (Nath et al., 1996a). We also proposed using the SBDP120 as a potential marker for apoptosis since several commercial anti- α -spectrin antibodies crossreact with rat and human α -spectrin (Nath et al., 1996a). In our recent study (Nath et al., 1998), we used caspase activation (against a peptidic substrate acetyl-DEVD-AMC) and the presence of SBDP120 to argue for the presence of an apoptotic component in both excitotoxin- (NMDA, kainate) and hypoxia/hypoglycemia-mediated neuronal death in cerebrocortical cultures. Independently, Du et al. (1997) also demonstrated that a caspase-3 like protease is activated in glutamate-challenged cerebellar granule neurons in culture.

In this study, we used a rat pup model of direct NMDA toxicity (McDonald et al., 1989). In parallel with traditional apoptosis markers (DNA laddering, TUNEL staining), we again sought evidence for caspase-3 activation by the two above-mentioned detection methods (caspase activity assay with peptidic substrate acetyl-DEVD-AMC and formation of SBDP120 on Western blot). We argue that using these biochemical approaches to earmark apoptosis greatly complements the more traditional techniques.

2. Materials and methods

2.1. NMDA-induced injury in rat pups

All animal experiments conform to the guidelines set by Parke-Davis and the Society for Neuroscience. Sprague-Dawley rat pups (Charles River Laboratories, Portage, MI) were used on postnatal day 6–7 (average 14 g). Animals were anesthetized by ethyl ether before 50 nmol of NMDA in the form of a saline solution (0.5 μ L of 7.35 mg/mL) or vehicle only was stereotaxically injected into the right posterior striatum with the aid of a stereotaxic platform. Caspase inhibitor is given i.p. (100 mg/kg) in a 5.5% DMF and methocel vehicle (50 μ L). Rat pups were allowed to recover for 2 h in an incubator kept at 36°C with a thermostat-controlled heating coil and then returned to their cages. After 24 h (unless otherwise stated), the pups were sacrificed and their cortical hemispheres were removed. The hemispheres were individually submerged in -70°C cooled isopentane. Frozen brains were powdered with pre-cooled mortar-pestle over dry ice. About 50 mg of the samples were added to 0.5 μ L of homogenization buffer containing 2% (w/v) SDS, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 10 μ g/mL 4-(2-aminoethyl)-benzenesulfonyl fluoride, 5 μ g/mL leupeptin, 10 μ g/mL pepstatin, 10 μ g/mL *N*-alpha-*p*-tosyl-L-lysine chloromethyl ketone and 10 μ g/mL *N*-alpha-*p*-tosyl-L phenylalanine chloromethyl ketone for 15–20 min to produce a clear lysate. One hundred μ L of trichloroacetic acid (100% (w/v)) was then added. The precipitated proteins were collected by centrifugation and washed with 1 mL of 2.5% trichloroacetic acid. The protein pellets were resolubilized with 50 μ L of 3 M Tris base and stored at -20°C until use (Wang et al., 1996).

2.2. Protein analysis

Protein concentration in tissue extracts was estimated with a modified Lowry assay (BioRad). Samples (15 μ g of protein) were run on SDS-PAGE (4–20% acrylamide) with a Tris-glycine running buffer system and then transferred onto a PVDF membrane (0.2 μ m) with Tris-glycine buffer system using a semi-dry electrotransferring unit (BioRad) at 20 mA for 1.5–2 h. The blots were probed with an anti- α -spectrin (monoclonal, Chemicon) antibody. The blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. Densitometric analysis of Western blots was performed using a color scanner (Umax UC630) and the NIH program Image 1.5.

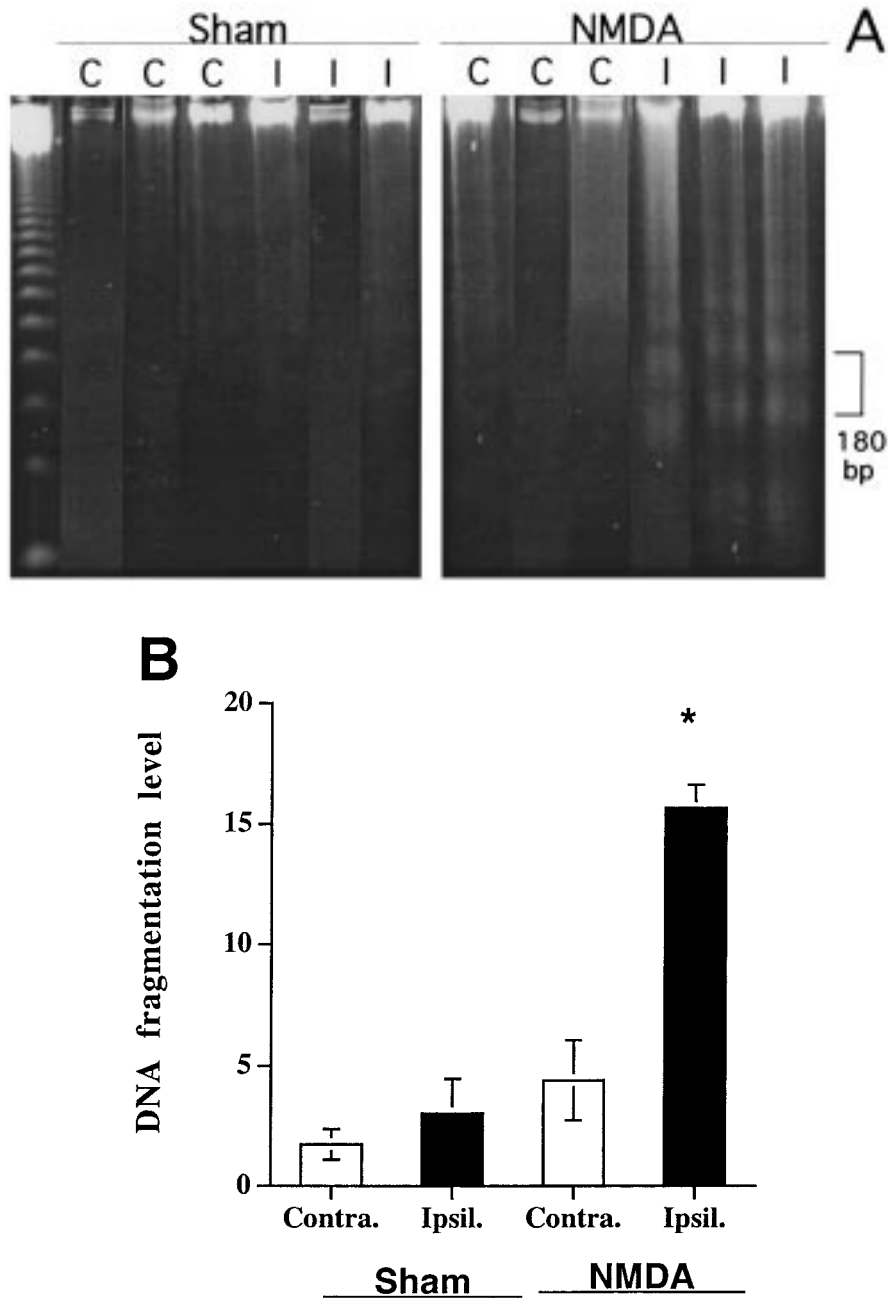


Fig. 1. DNA laddering on the ipsilateral hemisphere in rat pups 24 h after NMDA injection. In (A), agarose gel electrophoresis of genomic DNA from sham-operated or NMDA-injured ipsilateral (I) or contralateral (C) hemispheres at 16 h after injection. The lanes to the far left are loaded with DNA standards. In (B), DNA fragmentation was quantified as a time course after NMDA injection. Shown are the levels of DNA fragmentation from the ipsilateral (closed bars) or contralateral (open bars) hemispheres. Data are means \pm SEM ($n = 3$). Data significantly different from contralateral are indicated by * ($P < 0.05$, ANOVA).

2.3. DNA laddering assay

The DNA was extracted from rat pup brain hemispheres using the Easy-DNA kit (Invitrogen). Briefly, the tissue was quick frozen in ice-cold isopentane and pulverized with mortar and pestle. The DNA was extracted using manufacturer's protocol

and analyzed on a 1.5% agarose gel with 5 $\mu\text{g}/\text{mL}$ ethidium bromide and electrophoresed at 2 V/cm overnight. The overall levels of DNA fragmentation on agarose gels was quantified by densitometry using a computer-driven image scanner (Umax UC630) and an image analysis software Image 1.5 (From NIH).

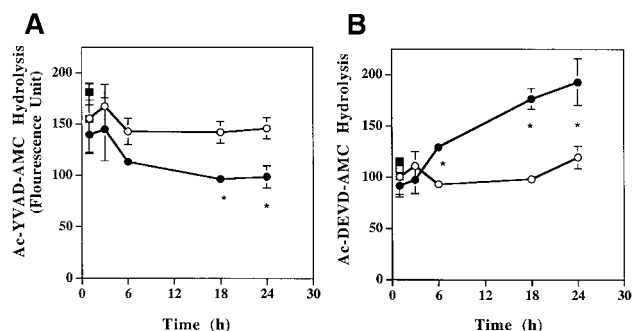


Fig. 2. Time course of caspase-1- and caspase-3-like activities towards peptide substrates after NMDA injection. Triton X-100 extracts (25 μ g protein) from (A) NMDA-injured (closed circles) or contralateral (open circles) hemispheres at 1, 3, 6, 18 and 24 h after injection were incubated in a reaction mixture with the caspase-1-preferred ac-YVAD-AMC peptide substrate (A), or the caspase-3-preferred ac-DEVD-AMC peptide substrate (B) (see Section 2). Data are means \pm SEM ($n = 3$). Data significantly different from contralateral are indicated by * ($P < 0.05$, ANOVA) or ** ($P < 0.02$).

2.4. Caspase activity assay

To assay for caspase-3-like protease, the ipsilateral and contralateral hemispheres of NMDA-injected rat pups were separately pulverized over dry ice and portions of the samples were lysed in a buffer containing 20 mM Tris-HCl (pH 7.4 at 4°C), 150 mM NaCl, 1 mM DTT, 5 mM EDTA, 5 mM EGTA and 1% (w/v) Triton X-100 for 90 min at 4°C. The centrifugation-cleared lysates were mixed with 50% (v/v) glycerol. Cell lysates (30 μ g protein) were assayed with 100 μ M acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (ac-DEVD-MCA; Bachem Bioscience), 100 mM Hepes, 10% glycerol, 1 mM EDTA, 10 mM DTT and 10 μ M Z-D-DCB (optional). Fluorescence (excitation 380 nm \pm 15 nm and emission 460 nm \pm 15 nm) was measured at 60 min with Cytoflor 2300 (Nath et al., 1996a).

3. Results

3.1. Early evidence of DNA fragmentation and condensation in NMDA-injured hemispheres

DNA laddering is among one of the more reliable techniques presently employed to study apoptosis both in vitro and in vivo. Thus, in the first set of experiments, we examined evidence of DNA fragmentation by electrophoresing genomic DNA samples from the NMDA-injected and contralateral hemispheres (24 h after injection) on agarose gel. As expected, very little DNA breakdown was detected in either hemispheres of sham-operated (vehicle-injected) animals (Fig. 1A). In contrast, ipsilateral hemispheres showed the presence of distinct DNA ladder in NMDA-injected rat

pups (Fig. 1A). We also quantified the levels of DNA fragmentation in these samples and found that DNA fragmentation significantly increased in the NMDA-injected hemisphere (Fig. 1B). We have also examined brain samples taken at other time points (8, 16, 24, 48 h) after NMDA injection. We found that small levels of DNA laddering began to appear on the ipsilateral side after 8 h (results not shown). By 16–24 h, the DNA fragmentation level in the ipsilateral side became the most intense. Significant increase of DNA laddering was also detected in the ipsilateral brain samples from the 48 h time point (results not shown). To address whether the DNA laddering signal came from neuronal or glial population, we next proceeded with TUNEL staining and counterstained with astrocyte marker GFAP of brain sections from NMDA-injected and sham-operated neonatal rat brains (1 day post injury). Ipsilateral neocortex in NMDA-injected rat pups showed widespread TUNEL-positive cells which are indeed neurons mostly. At higher concentrations, we noted that about 30% TUNEL-positive neurons showed highly condensed chromatin fragments within the nuclei, consistent with apoptotic phenotype (data not shown).

3.2. Evidence of caspase activation

To approach the apoptosis issue biochemically, we took advantage of the following two observations: (i) caspase-3 activation is linked to processing of caspase-3; and (ii) only processed form of caspase-3 can cleave peptidic substrates, such as ac-DEVD-AMC (Posmantur et al., 1997). We prepared Triton-X100 extracts from pulverized frozen hemispheres. We then subjected the extracts (equalized by protein amount) to a caspase activity assay. It utilized two synthetic substrates: ac-YVAD-AMC which is rapidly hydrolysed by caspase-1 but not caspase-3; ac-DEVD-AMC, on the other hand, which is rapidly hydrolyzed by caspase-3 but is also a substrate for caspase-1. In this experiment, we examined the possible elevation of caspase-1 or -3 activity during a time-course post-NMDA injection. At 1 and 3 h, the levels of ac-YVAD-AMC and ac-DEVD-AMC hydrolytic activities are present in both ipsilateral and contralateral hemispheres (Fig. 2A and 2B). But no significant difference between the two hemispheres was detected. By 18 and 24 h, there is actually a slight decrease in ac-YVAD-AMC hydrolysis in the NMDA-injected hemisphere (Fig. 2A). On the other hand, ac-DEVD-AMC hydrolysis in the NMDA-injured cortex was increased as early as 6 h and also at 18 and 24 h (Fig. 2B). Since no caspase-1 activation was observed (based on the lack of increase of ac-YVAD-AMC hydrolysis) (Fig. 2A), we attributed the increase in ac-DEVD-AMC activity to activated caspase-3 in the NMDA-injured

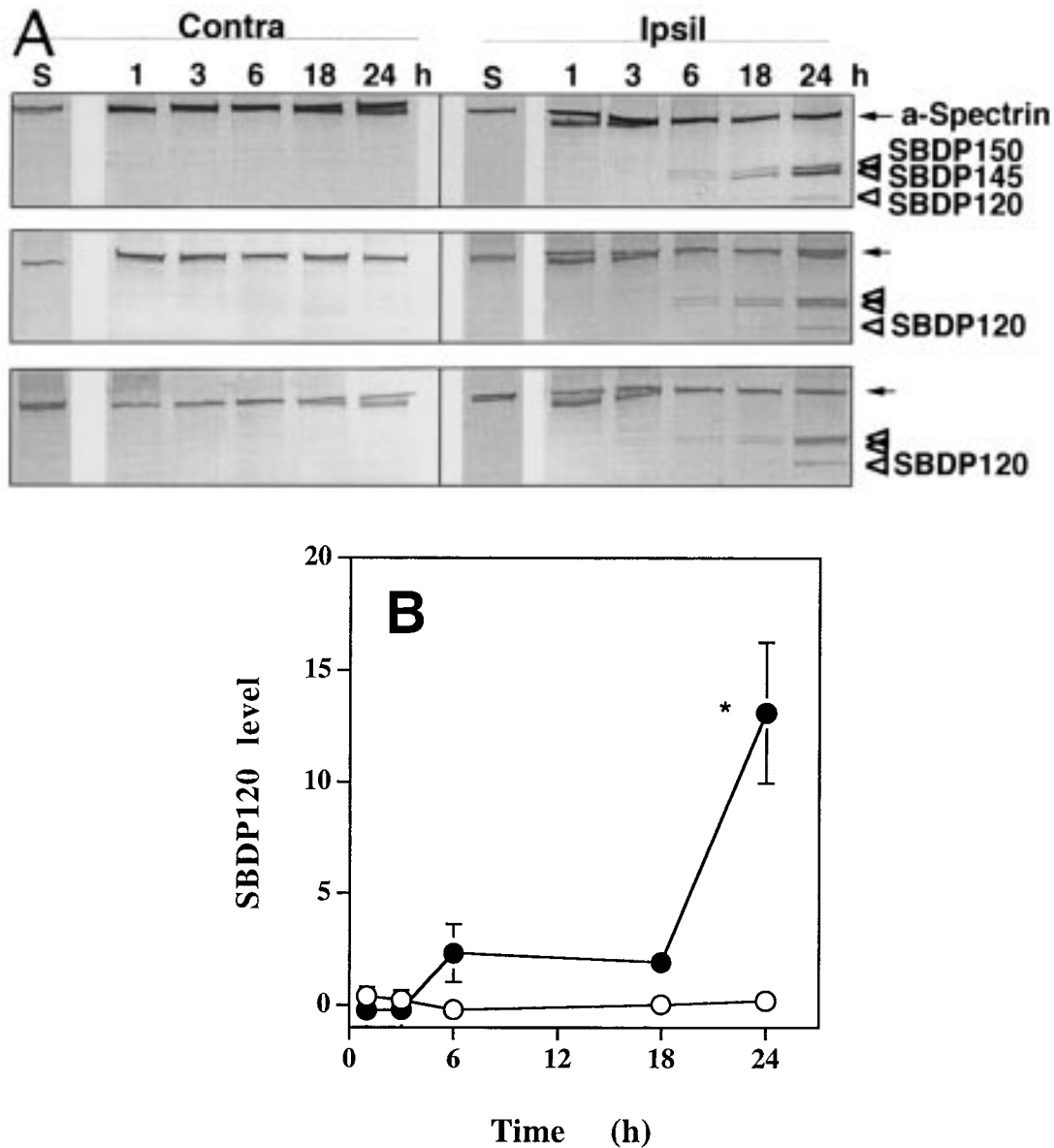


Fig. 3. Appearance of apoptosis-specific 120 kDa α -spectrin breakdown product in the NMDA injected hemisphere. Immunoblot analysis of α -spectrin breakdown of rat pup samples from (A) sham-operated (S), or NMDA-injured ipsilateral (ipsil) or contralateral (contra) hemispheres at 1, 3, 6, 18 and 24 h after injection. The intact α -spectrin is indicated by an arrow (280 kDa), the SBDP120 was indicated with open triangle, while SBDP150 and SBDP145 were indicated by double open triangles. Three individual samples from each time points are shown. (B) Quantification of the level of apoptosis-specific SBDP120 in samples from the ipsilateral (closed circles) or contralateral (open circles) hemispheres. Data are means \pm SEM ($n = 3$). Data significantly different from contralateral are indicated by * ($P < 0.05$, ANOVA).

hemisphere. Contralateral hemisphere showed no increase in either ac-DEVD-AMC or ac-YVAD-AMC hydrolytic activity. Taken together, data in Fig. 2 showed that increase of caspase-3 or a caspase-3 like activity is observed 6–24 h in the ipsilateral hemisphere following NMDA injection.

3.3. Formation of α -spectrin breakdown product SBDP120 in the NMDA-injured hemisphere

To further monitor caspase-3 activation, we exam-

ined the integrity of one of its best established substrates, α -spectrin. In the sham-operated animals, protein extracts from both hemispheres showed only intact α -spectrin (280 kDa), demonstrating that our extraction method did not introduce artificial post-mortem spectrin degradation, as we have previously established (Wang et al., 1996) (Fig. 3A). In the NMDA-injured group, we noted that spectrin breakdown products (SBDPs) began to emerge as early as 6 h and became prominent by 24 h (Fig. 3A). Three different fragments (bearing molecular weights of 150,

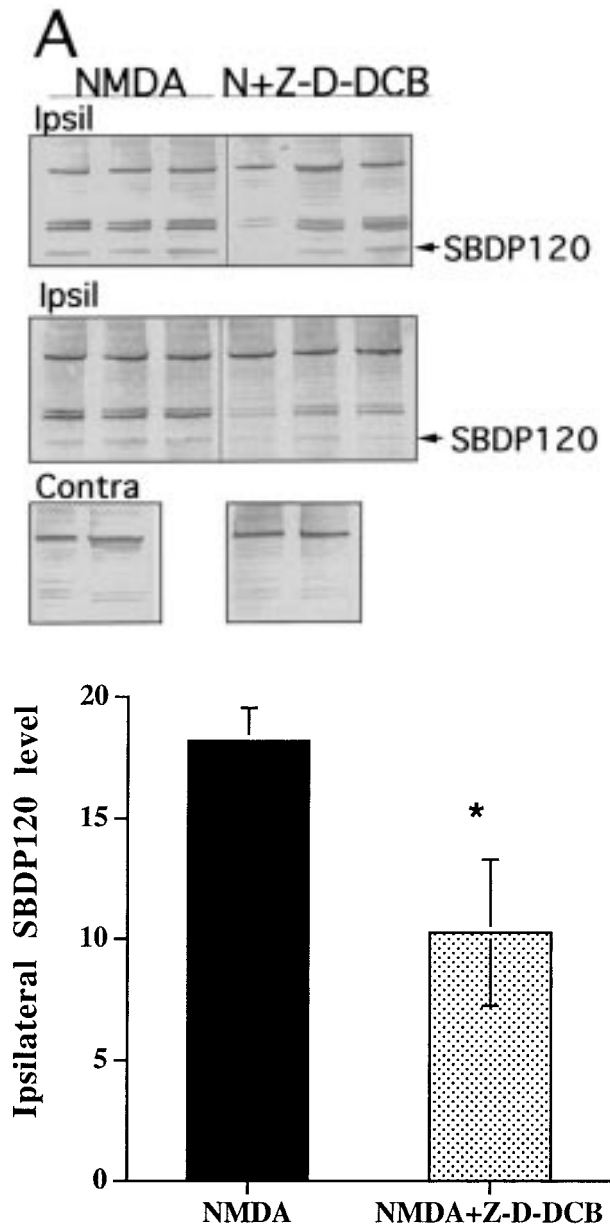


Fig. 4. Caspase inhibitor Z-D-DCB attenuated SBDP120 in NMDA-injured brains. (A) α -Spectrin breakdown in the ipsilateral (ipsil) and contralateral (contra) brain hemispheres in rat pups 24 h after NMDA treatment (NMDA) or NMDA treatment with 100 mg/kg of Z-D-DCB i.p. injection 30 min after NMDA injection was monitored (N+Z-D-DCB) as described in Section 2 (six pups for each group). Each lane represents protein sample (15 μ g) from an individual rat pup. The solid arrows indicate the apoptosis-specific SBDP120. (B) Densitometric quantification of SBDP120 in the ipsilateral hemisphere from either NMDA (open bar) or NMDA+Z-D-DCB treated (solid bar) rat pups was shown. Data are means \pm SEM ($n = 6$). Data significantly different from NMDA alone are indicated by * ($P < 0.05$, Student's t -test).

145 and 120 kDa) are observed in the ipsilateral hemispheres from the NMDA treatment group. We have previously shown that in cultured neurons, necrosis leads to calpain-mediated SBDP150 and SBDP145

whereas apoptosis produces the calpain-mediated fragments as well as the caspase-3-mediated SBDP150 and SBDP120 (Nath et al., 1996a,b). Therefore, based on molecular weight separation, the SBDP120 is an unambiguous marker for caspase-3 activation. We also quantified the level of SBDP120 against a time course after NMDA injury. In fact, mirroring the increase of caspase-3 activity (Fig. 3B), we noted that the level of caspase-3 specific SBDP120 began to rise at a low level by 6 h, reaching maximal level at 18 and 24 h in the ipsilateral hemispheres (Fig. 3A and 3B). Again the contralateral hemispheres showed little SBDP120 over the same time course (Fig. 3A and 3B) ($n = 3$).

To further ascertain that the SBDP120 in NMDA-injured brains is caspase-mediated, we examined the effects of caspase inhibitor Z-D-DCB on the levels of NMDA-induced SBDP120. We administered a group of NMDA-treated rat pups a bolus dose (100 mg/kg in saline, i.p.) of the low molecular weight (M.W. 454.3), Z-D-DCB or vehicle alone 30 min after NMDA injection. The i.p. route was used since the small size of the rat pups excluded the possibility of i.v. administration. We have previously shown that Z-D-DCB is very effective in blocking the caspase-3-mediated SBDP120 in apoptotic neurons in culture (Nath et al., 1996a). In this experiment, the protein samples were taken from the 24 h time point. Again, we noted significant levels of SBDP120 in the NMDA-injured hemispheres in the vehicle treatment group (Fig. 4A and 4B). On the other hand, in the Z-D-DCB treatment group, the ipsilateral hemispheres showed a partial but significant attenuation of the SBDP120 levels (Fig. 4A and 4B). This suggests that SBDP120 formed in this in vivo model of excitotoxicity is derived from a caspase or caspase-like activity.

4. Discussion

In the rat pup model of excitotoxicity, we report evidence of DNA laddering in the ipsilateral hemispheres, 16–24 h after the NMDA injection (Fig. 1). This echoes several recent reports showing similar evidence of apoptosis in a kainate toxicity model (Filipkowski et al., 1994) as well as in focal ischemia models in which excitotoxicity was implicated (Chen et al., 1997; Heron et al., 1993; Li et al., 1995a). But we extended these findings by showing that the apoptosis-linked caspase-3/7 like protease was activated, as evidenced by (i) increase in hydrolytic activity of caspase-3 substrate acetyl-DEVD-AMC in the ipsilateral hemisphere (Fig. 2) and (ii) the formation of caspase inhibitor-sensitive SBDP120 (Figs. 3 and 4). Based on a previous report that caspase-7 m-RNA is absent in neurons, it is most likely that caspase-3 is mediating the spectrin breakdown observed in NMDA-injured neurons. It is

important to point out that SBDP120 preceded the loss of brain mass, which usually takes 1–5 days. In the current study, we employed an anti- α -spectrin that detects both the intact protein as well as various fragments including SBDP120. It was an adequate tool since we performed Western blots analysis, which provided resolution of the various fragments. We also established that the majority of TUNEL-positive cells are neurons rather than astrocytes (results not shown), thus giving us the confidence with the notion that the SBDP120 observed mainly derived from the neuronal population. We have recently identified the N-terminal sequence of the SBDP120 (Wang et al., 1998). We reason that it would be ideal to generate a neo-N-terminal-specific antibody which can be employed in immunohistochemical staining and other qualitative and quantitative analysis of caspase activity in vivo (Wang et al., unpublished work). As caspase-3 is only activated in apoptosis but not necrosis (Armstrong et al., 1997), we propose that it will be a powerful yet simple technique to positively identify individual cells that are dying from apoptosis, as opposed to the more elaborated morphological identification by electron microscopy. Work is now in progress in our laboratory to produce SBDP120-specific antibodies.

Lastly, several recently available caspase-specific inhibitors (such as Z-D-DCB, Z-VAD-cmk, ac-YVAD-cmk, Z-VAD-fmk) are found to be powerful neuronal apoptosis inhibitors (Hara et al., 1997; Loddick et al., 1996; Nath et al., 1996a, 1996b; Schulz et al., 1996). Also they are ineffective against necrosis (Armstrong et al., 1997). Recently caspase inhibitors (Z-VAD-fmk, Z-DEVD-fmk) have been shown to produce neuroprotective effects in both a focal ischemia model as well as a traumatic brain injury model (Endres et al., 1998; Yakolev et al., 1997). A note of caution is that these inhibitors are also capable of inhibiting caspase-1. Beside its potential role in certain forms of apoptosis (Kuida et al., 1995; Li et al., 1995b), caspase-1 is also the established pro-interleukin 1 β -converting enzyme (Thornberry et al., 1992). Thus, pharmacological studies using general caspases inhibitors could also offer neuroprotection by suppressing IL-1 β -mediated neuro-inflammation component after ischemia (Schielke et al., 1998). On the other hand, Namura and et al. (1998) recently showed evidence of caspase activation in a focal ischemia model in mouse by using TUNEL, DEVD-based peptide substrate hydrolysis and positive staining with an antibody specific to the activated form of caspase-3. Their data and techniques are in agreement and complement the data presented here. In conclusion, these recent studies including the present study show evidence for an “apoptotic” component in a given form of neuronal injury involving excitotoxicity. It also raises the intriguing possibility that more powerful and selective cas-

pase inhibitors may have a therapeutic value in the treatment of various excitotoxin-mediated neurodegenerative disorders.

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