Calpain Facilitates the Neuron Death Induced by 3-Nitropropionic Acid and Contributes to the Necrotic Morphology

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Abstract. 3-Nitropropionic acid (3NP), an irreversible inhibitor of succinate dehydrogenase, has been used to model features of neurodegenerative disorders including Huntington disease, as well as acute neuronal insults such as cerebral ischemia. 3NP induces rapid necrosis and delayed apoptosis in primary cultures of rat hippocampal neurons. Low levels of extracellular glutamate shift the cell death mechanism to necrosis, whereas antagonism of NMDA receptors results in predominately apoptotic death. In the present study, the involvement of calpain proteases in the morphologic and biochemical alterations accompanying 3NP-induced neuron death was investigated. Immunoblots of spectrin breakdown products indicated Ca\(^{2+}\)-dependent calpain protease (calpain) activation within the 8 hours of 3NP administration, whereas caspase-3 activation was not evident until 16 to 48 hours after treatment. The NMDA receptor antagonist MK-801 (dizocilpine) decreased 3NP-induced calpain activity, but did not alter caspase-3 activity. Similar to MK-801, calpain inhibitors (Z-Val-Phe-H and Z-Leu-Phe-CONHEt) shifted the cell death morphology towards apoptosis and delayed, but did not prevent, the 3NP-induced cell death. Together, the results indicate that following 3NP administration, increased calpain activity precedes caspase-3 activation, contributes to the necrotic morphology, and facilitates and accelerates the cell death.

Key Words: Apoptosis; Excitotoxicity; Huntington disease; Necrosis; N-methyl-D-aspartate; Spectrin.

INTRODUCTION

The succinate dehydrogenase inhibitor 3-nitropropionic acid (3NP), an irreversible inhibitor of succinate dehydrogenase, has been used to model features of neurodegenerative disorders including Huntington disease, as well as acute neuronal insults such as cerebral ischemia (1). This is relevant to age-related neurodegenerative disorders such as Huntington disease, in which bioenergetic defects are implicated (2), and to acute insults such as cerebral ischemia. Indirect excitotoxic mechanisms are strongly implicated in 3NP-toxicity (3), and 3NP-induced necrosis is exacerbated by low levels of extracellular glutamate. The N-methyl-D-aspartate receptor antagonist dizocilpine maleate (MK-801) delays, but does not prevent, 3NP-induced neuron death and shifts the cell death morphology to apoptosis (1, 4). Similarly, MK-801 has been shown to unmask apoptotic mechanisms following oxygen-glucose deprivation (5).

Two families of nonlysosomal cysteine proteases are likely involved in the neuron death induced by 3NP: caspases and calpains. Caspases are a family of cysteine proteases that preferentially cleave after aspartate residues in the target proteins (6) and participate in a proteolytic cascade culminating in apoptotic cell death (7, 8). The essential contribution of caspase activation to apoptotic morphology, particularly nuclear fragmentation, is well documented (7, 9–12). Calpains are a family of calcium-activated neutral cysteine proteases (13) that are implicated in neuronal injury resulting from altered Ca\(^{2+}\) homeostasis during excitotoxic insults and are activated during necrotic death (14–18), but are also activated during some forms of apoptosis (16–18). Elevated intracellular Ca\(^{2+}\) also results in the activation of additional enzymes, including nitric oxide synthetase, endonucleases, calcineurins, and protein kinases (19). Thus, although calpain is clearly activated during necrosis and some forms of apoptosis, its contribution to cell death morphology is unclear.

Both calpains and caspases are active in some forms of neuron death such as glutamate toxicity, oxygen-glucose deprivation, or stretch-induced neuron death (20–22). Apoptosis and necrosis are thought to represent distinct cell death mechanisms, exhibiting different morphological, biochemical, and pharmacological characteristics (23, 24). Mild-to-moderate toxic insult often results in apoptotic death while a more severe insult causes necrosis (25–27). However, it has also been suggested that apoptosis and necrosis may represent the extreme ends of a cell death continuum rather than distinct cell death mechanisms (28–30), or that there are alternate cell death pathways with characteristics of both apoptosis and necrosis (31, 32). It has not yet been determined whether neuron death observed in vivo following acute insults such as cerebral ischemia represents classical apoptosis or necrosis, or has characteristics of both pathways (33, 34). The activation of calpains in both apoptotic and necrotic cell death may be at least partially responsible for the morphologic continuum observed previously (28).

Calpains and caspases both cause limited proteolysis of target proteins, sharing many substrates including non-erythroid α-spectrin (35–38). The contribution of these
cysteine proteases to the morphologic and biochemical alterations accompanying 3NP-induced neuron death was investigated in the present study.

MATERIALS AND METHODS

Materials

3NP was purchased from Aldrich (Milwaukee, WI). Propidium iodide (PI), poly-D-lysine, trypsin, mung bean trypsin inhibitor, pepstatin A, and cycloheximide were from Sigma (St. Louis, MO). Hoechst 33342 and calcine AM were from Molecular Probes (Eugene, OR). Hank’s Balanced Salt Solution, Neurobasal medium, B27 supplement, glutamine, and glutamate were purchased from Life Technologies (Rockville, MD). The calpain inhibitors Benzoyloxy carbonyl-Val-Phe-H (Z-VEH); MDL28170, (Calpain Inhibitor III) and Benzoyloxy carbonyl-Val-Phe-CONHCH₂CH (Z-LECONHET) were synthesized at Cephalon (West Chester, PA) by Patrician Mesina and Ron Bihovsky, respectively, according to published procedures (39, 40), with some additional Z-VEH purchased from Calbiochem (San Diego, CA). The caspase inhibitor Benzoyloxy carbonyl-Val-Ala-Asp-2,6-dichlorobenzoate (Z-VDADCB) was synthesized at Cephalon by Manoj Das according to Dolle et al (41). Compounds were purified by HPLC and their structures confirmed by NMR. The caspase inhibitor Benzoyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) was purchased from Enzyme Systems Products (Livermore, CA). The noncompetitive NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate was from Research Biochemicals International (Natick, MA). Phenylmethanesulfonyl fluoride, leupeptin, and the in situ cell death detection kit were from Roche Molecular Biochemicals (Indianapolis, IN). For Western blots, a monoclonal antibody against α-spectrin, mAb 1622, was obtained from Chemicon (Temecula, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG was from Zymed (S. Louis, MO). Hoechst 33342 and the in situ cell death detection kit were from Roche Molecular Biochemicals (Indianapolis, IN). For Western blots, a monoclonal antibody against α-spectrin, mAb 1622, was obtained from Chemicon (Temecula, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG was from Zymed (San Francisco, CA). Nitrocellulose membranes were from Bio-Rad (Hercules, CA). Detection was via chemiluminescence, using the Pierce SuperSignal West Pico chemiluminescent substrate (Rockford, IL) and Kodak T-Max film (Rochester, NY).

Primary Hippocampal Neuronal Cultures

Primary cultures of fetal rat (E18) hippocampal neurons were prepared as described previously (1). Cells were maintained in the serum-free Neurobasal media containing 2% B27 at 37°C in a humidified incubator with 6% CO2 and 94% air. Seven-days-in vitro (DIV) cultures were used throughout the experiments. Treatment was initiated by replacing the culture media with fresh media containing drugs or chemicals. Cultures were preincubated with the protease inhibitors for 2 h before the 3NP treatment. Neuronal survival was assessed by counting viable cells on phase-contrast micrographs taken before and after treatment. Viability was determined by morphological criteria, including smooth cell body, intact neurites, and phase-bright soma. Cell viability was confirmed using double staining on unfixed cultures with propidium iodide (PI) and calcine AM, or PI and Hoechst 33342. Primary hippocampal cells were incubated in 5 μM calcine AM and 5 μg/ml of PI in Neurobasal media at 37°C. After 45 min, the cells were washed 3 x with PBS, Vectashield fluorescent mounting media (Vector Laboratories, Inc., Burlingame, CA) was added and dishes were coverslipped. For quantification, 200 cells/dish were randomly counted and percentage of surviving (PI-negative, calcine AM-positive) and dead cells (PI-positive) were calculated.

Analysis of Apoptosis and Necrosis

The procedures for quantifying different forms of cell death (apoptosis and necrosis) are similar to those described previously (1). Brieﬂy, cells were ﬁxed with 4% paraformaldehyde in phosphate buffered saline (PBS, Na2HPO4, 72 mM; NaH2PO4, 28 mM; NaCl, 0.9%; pH 7.2), stained with PI (5 μg/ml in PBS containing 0.1% Triton X-100), and visualized under an epifluorescent microscope (Olympus BH2 or AX70) with the filter set for rhodamine. Living cells displayed cytoplasmic staining and large, oval-shaped, evenly stained nuclei. Apoptotic cells were identiﬁed by the presence of nuclei with fragmented, condensed chromatin (apoptotic bodies). Necrotic/non-apoptotic cells were identiﬁed by small, condensed nuclei without apoptotic bodies. The percentages of apoptotic and necrotic nuclei per total nuclei (≥500 cells per culture dish) were calculated. In some cases, unfixed cells were stained with the permeant dye Hoechst 33342 and the membrane impermeant PI for 15 min and fluorescent photomicrographs were obtained using a triple band filter. The criteria for apoptotic and necrotic death were as described for PI staining in ﬁxed, permeabilized cells.

Immunoblotting

Non-erythroid α-spectrin and its breakdown products were detected with monoclonal antibody 1622 (Chemicon). After treatments, cells were rinsed with PBS containing 1 mM phenylmethanesulfonyl fluoride (PMSE; Calbiochem), resuspended with lysis buffer (62.5 mM Tris (pH 6.8), 6M urea, 10% glycerol, and 2% SDS), scraped, the lysate collected, and the samples sonicated. Protein content was determined using the BCA protein Assay (Pierce). 2-Mercaptoethanol (5% w/v) and 0.04% bromophenol blue were added and the samples were denatured at 65°C for 30 min. Samples containing 10 μg protein were separated by SDS-PAGE using 6.5% polyacrylamide gels, along with molecular weight markers. Following SDS-PAGE, polypeptides were transferred electrophoretically onto 0.45-μm nitrocellulose membranes. Membranes were incubated at room temperature for 60 min with 5% nonfat milk in 50 mM Tris-saline containing 0.05% Tween-20, pH 7.5 (TTBS). The blots were incubated in the primary antibody (mAb 1622, 0.1 μg/ml) overnight in TTBS at room temperature. The blots were rinsed, incubated in horseradish peroxidase-conjugated goat anti-mouse IgG (1:3,000) for 60 min, rinsed, and developed using Pierce SuperSignal West Pico chemiluminescent substrate and then exposed to Kodak T-MAX film. All blots were replicated a minimum of 3 x using different culture preparations.

Statistical Analysis

Results are expressed as the mean ± SEM, with each replicate representing a separate culture preparation. A minimum of 3 cultures per preparation were used in each experiment. Tests for statistical significance included Student t-test or ANOVA.
followed by Scheffe’s F-test for comparison of multiple experimental conditions.

RESULTS

Spectrin Proteolysis

Although 3NP administration to primary rat hippocampal neurons was previously shown to result in both necrotic and apoptotic death, the profile of caspase and calpain activation has not been determined. Non-erythroid α-spectrin is a sensitive substrate of both μ-calpain and caspase-3 (35, 36, 42). Calpain-mediated proteolysis of α-spectrin results in 145- and 150-kDa spectrin breakdown products (SBDPs), while caspase-3 proteolysis results in breakdown products of 150 and 120 kDa (35). Thus, the 145-kDa SBDP is indicative of calpain activity, the 120-kDa SBDP indicates caspase-3 activity, and the 150-kDa SBDP could result from calpain and/or caspase activation.

In control neurons, a monoclonal antibody against the carboxyl terminal region of α-spectrin (mAb 1622, Chemicon) detected a 280-kDa band representing intact α-spectrin (Fig. 1). Bands of lower molecular weight, including 150, 145, and 120 kDa, were evident but at weak intensity. Administration of 5 mM 3NP resulted in the appearance of the 145- and 150-kDa SBDPs within the first 8 h of treatment (Fig. 1A). The 120-kDa SBDP, indicative of caspase-3 activity, was detected 48 h following treatment (Fig. 1A). With longer film exposure, faint 120-kDa bands could be observed at 16 h and 24 h (results not shown). In neurons treated with 3NP (5 mM) plus the NMDA receptor antagonist MK-801 (10 μM), the intensity of the 145- and 150-kDa SBDPs was decreased, while the temporal profile of the SBDPs was similar to that observed in 3NP-treated cultures (Fig. 1B, D). In neurons treated with 3NP plus low levels of extracellular glutamate (10 μM), the temporal profile of spectrin breakdown was similar to that observed with 3NP alone. The 145- and 150-kDa SBDPs were evident 8 h after 3NP administration, but the 120-kDa band was not prominent until 48 h (Fig. 1C, D).

To facilitate comparison of the spectrin breakdown that occurs under the various treatment conditions (3NP, 3NP plus glutamate, 3NP plus MK-801), a separate set of 48-h samples were examined together on the same blot (Fig. 1D). The results demonstrate a similar intensity of the 120-kDa band with each of the treatment conditions. In contrast, MK-801 diminished the intensity of the 145- and 150-kDa SBDPs as compared to cultures treated with 3NP in the presence or absence of added glutamate.

To examine the effects of calpain and caspase inhibition, we focused on conditions resulting in predominantly necrotic (3NP and glutamate) or apoptotic (3NP and MK-801) neuron death. In hippocampal neurons treated with 3NP and glutamate for 48 h, the broad-spectrum caspase inhibitor Z-VAD.FMK (Fig. 2A) did not block the appearance of the 145- and 150-kDa SBDPs, but prevented the increase in intensity of the 120-kDa band. Similar results were obtained with Z-VAD.DCB (results not shown). Calpain inhibition with Z-VFH greatly diminished the intensity of the 145-kDa and, to a lesser extent, 150-kDa SBDPs. The faint 150-kDa band was also evident following combined administration of Z-VAD.FMK and Z-VFH (results not shown). The presence of the 150-kDa SBDP may reflect incomplete calpain or caspase inhibition or, possibly, α-spectrin proteolysis by additional proteases (43).

Similar results were obtained in neurons treated with 3NP plus MK-801 (Fig. 2B). Z-VAD.FMK blocked the appearance of the 120-kDa band, but not the 145- and 150-kDa SBDPs. Calpain inhibition with Z-VFH reduced the intensity of the 145- and 150-kDa SBDPs but not the 120-kDa band.

Neuron Survival and Morphology

Pretreatment of hippocampal neurons with the calpain inhibitor Z-VFH attenuated neuron death after 6 h and 24 h, but not after 48 h, of exposure to 5 mM 3NP (Fig. 3A). In contrast, the caspase inhibitor Z-VAD.FMK did not alter the time course or magnitude of cell death (Fig. 3A). Combined calpain and caspase inhibition also delayed, but did not prevent, 3NP neuron death (Fig. 3A).

In cultures treated with 3NP plus MK-801, the broad-spectrum caspase inhibitors Z-VAD.DCB and Z-VAD.FMK did not prevent the cell death, but did attenuate the apoptotic morphology (Fig. 3B–E). Both inhibitors reduced the percentages of cells containing nuclear apoptotic bodies, with a complementary increase in the number of cells with unfragmented but shrunken and sometimes irregularly shaped nuclei (quantified as non-apoptotic nuclei) (Fig. 3C–E). These morphologically unusual nuclei were not seen in the control cultures and cultures treated with 3NP plus MK-801 alone. Most of these unusual nuclei were stained with PI in unfixed cultures, indicating a loss of cell viability. The caspase inhibitors per se were not toxic to the cultured hippocampal neurons during the course of treatment and they had no effect on the necrotic cell death induced by 3NP plus glutamate (data not shown). Z-VAD.FMK can inhibit calpain activity at high concentrations and with prolonged incubation (44), however, this was not observed in the present study.

Similar to the results obtained in 3NP-treated neurons, pretreatment of the cells with the calpain inhibitors Z-VFH or Z-LFCONHet significantly reduced the acute cell death induced by 5 mM 3NP plus 10 μM glutamate, but had little effect on the delayed cell death (Fig. 4A). The calpain inhibitors also altered the morphology of cell death, significantly reducing the percentage of necrotic cells and increasing the number of apoptotic cells (Fig. 4B).
Fig. 1. Effects of 3NP on spectrin breakdown. After exposure to 5 mM 3NP (A), 3NP plus 10 μM MK-801 (B), or 3NP plus 10 μM L-glutamate (C) for the duration indicated, primary cultures of rat hippocampal neurons were processed for Western blots of α-spectrin breakdown as described in Materials and Methods. The antibody used (mAb 1622, Chemicon) recognizes the carboxy terminus of α-spectrin. Intact α-spectrin is 280 kDa. Calpain-mediated proteolysis results in spectrin breakdown products (SBDPs) of 150 and 145 kDa, which were prominent after 8 h in neurons treated with 3NP (A) or 3NP plus glutamate (B), but were much less intense when MK-801 was administered along with 3NP (C). Proteolysis of α-spectrin by caspase-3 results in SBDPs of 150 and 120 kDa. The 120-kDa band was not prominent until 48 h of 3NP exposure. Direct comparison of neuron exposure to the various treatment conditions for 48 h (D) illustrates that all treatments resulted in a similar magnitude of caspase-3 activation, indicated by the 120-kDa SBDP, but varying intensities of the 145- and 150-kDa SBDPs, which largely reflects calpain activity.

4B). Calpain inhibitors alone did not induce cell death (data not shown).

DISCUSSION

The goal of the present study was to determine the involvement of cysteine proteases, calpains, and caspases in the 2 modes of cell death induced by 3-nitropropionic acid (3NP), necrosis and apoptosis, respectively. Previously, based on morphological criteria, 3NP was shown to induce both apoptotic and necrotic cell death (1). Necrotic cell death was exacerbated by low concentrations of extracellular glutamate, while the NMDA receptor antagonist MK-801 attenuated the necrotic morphology and shifted the cell death mechanism to apoptosis.

Role of Calpain

Excessive activation of calpains has been proposed as a contributing factor in ischemic neurodegeneration and neurotrauma. Calpains, particularly μ-calpain, are clearly activated during insults resulting in necrotic death, such as NMDA-mediated excitotoxicity (14), but their role in cell death is less clear. Calpain inhibition is neuroprotective in some studies (45–48), but not in others (49, 50).

In the present study, calpain activity was prominent in the cell death induced by 3NP in the presence or absence of added glutamate. Calpains were activated prior to caspases, and calpain inhibition delayed neuron death induced by 3NP. Calpain inhibition also altered the morphology of cell death, decreasing the percentage of
Fig. 2. Effects of calpain and caspase inhibitors on 3NP-induced spectrin breakdown. A: Effects of inhibitors on spectrin breakdown in cultures treated for 48 h with 5 mM 3NP plus 10 μM glutamate (A) or 5 mM 3NP plus 10 μM MK801 (B) in the presence or absence of calpain (Z-LF.H, 25 μM) or caspase (Z-VAD.FMK, 25 μM) inhibitors that were added 2 h before 3NP. Treatment with 3NP plus glutamate (A) resulted in the appearance of spectrin breakdown products (SBDPs) of 150, 145, and 120 kDa. Pretreatment with the caspase inhibitor Z-VAD.FMK prevented the appearance of the 120-kDa SBDP but had little effect on the 145- and 150-kDa bands. In contrast, pretreatment with Z-VF.H resulted in the almost complete disappearance of the 145-kDa band, decreased intensity of the 150-kDa SBDP, but increased intensity of the 120-kDa SBDP. In neurons treated with 3NP plus MK801 (B), SBDPs of 145/150 and 120 kDa are evident. Caspase inhibition with Z-VAD.FMK markedly attenuated the 120-kDa SBDP but not the 145- and 150-kDa bands. Calpain inhibition with Z-VF.H attenuated the 145- and 150-kDa bands, but had little effect on the 120-kDa SBDP.
Fig. 3. Effects of calpain and caspase inhibitors on the magnitude and morphology of 3NP-induced neuron death. Primary cultures of rat hippocampal neurons were treated with 3NP for the duration indicated, in the presence or absence of the caspase inhibitor Z-VAD.FMK and the calpain inhibitor Z-LF (A). Cell death was determined using propidium iodide and calcein-AM (see Materials and Methods). In a separate set of experiments, the morphology of cell death was evaluated in cultures treated with 5 mM 3NP plus 10 μM MK-801, in the presence or absence of the caspase inhibitors Z-VAD.DCB or Z-VAD.FMK (B). To evaluate cell death morphology, the cultures were stained with propidium iodide and Hoechst 33342. At 48 h after treatment with 3NP plus MK-801, neuronal death and the percentages of cells with apoptotic (fragmented) nuclei and nonapoptotic (includes necrotic cells and other dead cells with irregular but unfragmented nuclei) were quantified. The results demonstrate that caspase inhibition results in a significant decrease in apoptotic neuron death, but a corresponding increase in nonapoptotic death, with no significant change in total neuron death. Control neurons are illustrated in panel (C). Panel (D) is a photomicrograph of neurons treated with 3NP plus MK-801 for 48 h. Panel (E) represents neurons treated with 3NP, MK-801, and Z-VAD.DCB. All cells are
Fig. 4. Effects of calpain inhibitors on neuron death induced by 3NP plus glutamate. Primary cultures of rat hippocampal neurons were treated with 5 mM 3NP plus 10 μM glutamate for the duration indicated, in the presence or absence of the calpain inhibitors Z-VF.H (25 μM) and Z-LFCONHet (25 μM), and cell viability was quantified (A). The percentages of apoptotic and necrotic cells were quantified in cultures 48 h following treatment with 3NP plus glutamate, with or without the calpain inhibitors (B). Panel (C) is a photomicrograph of neurons treated with 3NP plus glutamate for 48 h and labeled with the membrane-permeant Hoeschst 33342. Panel (D) represents neurons treated with 3NP plus glutamate and the calpain inhibitor Z-VF.H. Apoptotic neurons possess fragmented nuclei with apoptotic bodies and necrotic/nonapoptotic neurons exhibit shrunken, irregular nuclei, as indicated in Figure 3. Calpain inhibition increased the percentage of neurons exhibiting apoptotic morphology and decreased the percentage of necrotic/nonapoptotic morphology with little effect on total neuron death. Data are mean ± SEM (n = 6). *Significantly different from treatments without calpain inhibitors; p < 0.01.

labeled by the membrane-permeant Hoeschst 33342 (blue), whereas staining with propidium iodide (pink) indicates cell death accompanied by a loss of membrane integrity. Many apoptotic nuclei (arrow) and shrunken, irregular but unfragmented nuclei (arrowhead) were labeled with propidium iodide. Scale bar = 25 μm. Data are mean ± SEM (panel A: n = 4; panel B: n = 6). *Significantly different from cultures without calpain or caspase inhibitors; p < 0.01.

nonapoptotic/necrotic neurons and increasing the percentage of apoptotic cells. Previously, we observed that the NMDA receptor antagonist MK-801 delayed 3NP-induced neuron death and shifted the death morphology to apoptosis. In the present study, MK-801 was also shown to decrease the magnitude of calpain activation. Thus, decreasing calpain activity by either pharmacologic inhibition or attenuating the elevation in intracellular Ca\(^{2+}\) produced similar results. Together, these results suggest that calpain activation facilitates 3NP-induced neuron death and contributes to the necrotic morphology.

The observation that calpain activation facilitates neuron death is consistent with the importance of many calpain substrates in cell survival (38, 51). Calpain substrates include cytoskeletal proteins, plasma membrane associated proteins, life-death proteins, signal transduction proteins, cell cycle proteins, and transcription factors (38). Calpain-mediated proteolysis of cytoskeletal proteins and plasma membrane-associated proteins is consistent with the altered morphology of organelles and plasma membrane damage associated with necrotic death. Caspases are the executioners of apoptosis (7), and calpains cleave many of the same or similar substrates (38). However, calpains do not cleave DFF45 (DNA fragmentation factor-45)/ICAD (inhibitor of caspase-activated DNase). DFF45/ICAD proteolysis by caspase-3 releases active DFF40/CAD, the endonuclease responsible for the nuclear fragmentation characteristic of apoptosis (11, 12).

The inability of calpain (or caspase) inhibition to protect against 3NP-induced cell death likely reflects the severe nature of this insult. 3NP is present in the culture media for the full duration of the treatment (up to 48 h). As an irreversible inhibitor of succinate dehydrogenase, 3NP results in a profound impairment of energy metabolism, making recovery unlikely. In contrast, where calpain inhibition has been demonstrated to be neuroprotective, the insults are of a more transient nature, such as excitotoxicity (47), oxygen-glucose deprivation (20), and cerebral ischemia (52). The investigation of oxygen-glucose deprivation by Newcomb-Fernandez et al (20) is the most relevant to the present study. Ten hours of oxygen-glucose deprivation followed by 12 h of reperfusion in septo-hippocampal cultures resulted in almost exclusive apoptotic neuron death associated with both calpain and caspase activity. Calpain inhibition (100 \(\mu\)M Z-VF\(\_H\)) markedly reduced levels of the 145- and 150-kDa spectrin breakdown products and also decreased cell death, as assessed by lactate dehydrogenase release. The caspase inhibitor DEVD (100 \(\mu\)M) also resulted in substantial calpain inhibition (145-kDa SBDP), but had only modest effects on caspase activity and neuron death. Similar to the results obtained in the present study, the Newcomb-Fernandez study demonstrates that calpain facilitates neuron death under conditions of combined calpain and caspase activation, but the influence of calpain and caspase inhibition on cell death morphology was not examined (20).

Role of Caspases

Caspase inhibition failed to prevent or delay neuron death induced by 3NP. This contrasts with several previous studies demonstrating neuroprotective effects of caspase inhibition against neuron death under a variety of conditions, including nerve growth factor withdrawal from sympathetic neurons (53), potassium deprivation of cerebellar granule cells (54), NMDA-induced apoptosis in cortical neurons (55), and 6-hydroxydopamine in nigral neurons (56). However, Z-VAD.FMK failed to prevent cell death in low-K\(^{+}\)-induced apoptosis in cerebellar granule cells (57) and briefly delayed, but did not protect, against 6-OHDA toxicity (58). The concentrations of Z-VAD.FMK used in this study are similar to those previously shown to block apoptosis and improve cell survival (59, 60) and to reduce caspase activity to levels below that observed in control neurons (61).

Caspase inhibitors, including Z-VAD.FMK, block many nuclear events associated with apoptosis as well as externalization of phosphatidylserine, but not membrane blebbing and the eventual cell death in several conditions in non-neuronal cells (62–64). In the present study, the caspase inhibitors failed to prevent 3NP-induced neuron death, but did block apoptotic morphology. This is consistent with results in non-neuronal cells in which caspase activation occurs after the commitment to cell death in chemical-induced apoptosis (65). Previous reports in neural and non-neural cells also describe a shift from apoptotic to necrotic death in the presence of caspase inhibitors (32, 58, 66, 67). Formigli et al (30) have described cell death induced by metabolic impairment in a rat fibroblastic cell line that has morphological and biochemical characteristics of both apoptosis and necrosis, and have referred to this as aponecrosis. Caspase inhibition shifted the cell death mechanism to necrosis, but the role of calpain was not examined.

Cross-Talk between Cysteine Proteases

Increasing evidence suggests a possible interaction between calpains and caspases in cell death (38), although there are contrasting reports regarding the nature of this interaction. Calpain can be an upstream regulator of caspase activation in both neuronal and non-neuronal cells (68–70).

Calpain can promote apoptosis by cleaving procaspase 12 to its active form (71), initial cleavage of procaspase 3 (68) or activation of caspase-3 (72), cleaving Bid and Bax to active proapoptotic fragments (73, 74), and cleaving the loop region in the anti-apoptotic Bcl-xl to convert this into a pro-apoptotic protein (71). In contrast, calpain activation is reported to inhibit the processing of procaspase-3 and–9 into their active forms (75) and may also.
inhibit apoptosis by degrading the apoptotic protease activating factor-1 (76). In addition to the regulation of caspase activity by calpains, caspase activation may promote calpain activity via cleavage of the calpain inhibitor protein calpastatin (77, 78).

The results obtained in the present study demonstrate that the time course and magnitude of caspase-3 activation, assessed by the presence and intensity of the 120-kDa SBDP, was similar under conditions of extensive (3NP plus glutamate) or mild (3NP plus MK801) calpain activity. Under conditions where 3NP-induced death is largely apoptotic (3NP plus MK-801), calpain inhibition did not alter the extent of cell death and did not inhibit caspase activity as assessed by spectrin breakdown. Under these conditions, caspase activation is neither calpain-dependent nor blocked by calpain activity. Under conditions where there is a large percentage of necrotic death (3NP plus glutamate), caspase inhibition did not alter calpain activity as assessed by spectrin breakdown. Calpain inhibition did result in a greater 120-kDa SBDP, suggesting the possibility of increased caspase activity. However, this may simply reflect increased substrate availability. There is a greater loss of α-spectrin in neurons treated with 3NP plus glutamate than with 3NP plus MK801. By attenuating calpain mediated-spectrin breakdown, more α-spectrin is available as a substrate for caspase-3. The results of the present study did not reveal a positive or negative interaction between calpain and caspase activity.

Summary

While the role of caspase activation in apoptotic cell death morphology is well characterized, the role of calpains is less clear. The results of this study indicate that the extent of calpain activation is a major determinant of the neuron death morphology under conditions in which both calpains and caspases are activated. Insults inducing high levels of calpain activity (3NP, 3NP plus glutamate) result in a large percentage of necrotic/nonapoptotic neuron death and a more rapid cell demise. In contrast, insults resulting in low levels of calpain activity (3NP plus calpain inhibitors, 3NP plus MK801) are associated with predominantly apoptotic neuron death. Wang proposed that calpain activation during apoptosis may have an auxiliary and augmentative role to caspase activation (38). The results from the present study provide experimental support for such a role and provide at least a partial explanation for the apoptotic-necrotic continuum described previously by Portera-Cailliau et al (28) in which excitotoxin-induced death resulted in morphologic profiles of neuron death with characteristics of both apoptosis and necrosis. Our results suggest that the magnitude of calpain activation in individual neurons strongly influences the extent of necrotic morphology.
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