Evidence That Inhibition of Cathepsin-B Contributes to the Neuroprotective Properties of Caspase Inhibitor Tyr-Val-Ala-Asp-Chloromethyl Ketone*

Received for publication, April 9, 2001, and in revised form, June 25, 2001

Published, JBC Papers in Press, June 26, 2001, DOI 10.1074/jbc.M1035150200

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During the use of tetrapeptide and other proprietary caspase inhibitors in the study of neurodegeneration, we had concluded that mechanisms other than the inhibition of caspases contributed to the protective effects of certain caspase inhibitors. Here we report our studies to identify a target for and hence a mechanism by which the tetrapeptide inhibitor tyrosine-valine-alanine-aspartate-chloromethyl ketone (Ac-YVAD-cmk) is able to rescue neuronal cell cultures from cell death. Ac-YVAD-cmk rescued neuronal cells from cell death in response to oxidative stress and oxygen/glucose deprivation. Affinity labeling with biotinylated YVAD-cmk demonstrated distinct binding proteins for the inhibitor in cells from the central nervous system versus Jurkat cells. Binding to the novel target protein was displaced by class-specific protease inhibitors and suggested that the target is a cysteine protease. Affinity purification and sequencing identified the target as cathepsin-B. Cathepsin-B inhibitors competed with biotinylated YVAD-cmk for the target protein. The availability of the target for binding was reduced in cells that had been rescued by unlabeled inhibitor. Cathepsin-B inhibitors rescue hippocampal slices from cell death induced by oxygen/glucose deprivation. These data provide evidence to support a role for cathepsin-B in neuronal cell death, particularly that following ischemia.

The mechanisms of cell death involved during development and in degenerative diseases are an area of intense study at present. Key among cell death mechanisms are those of necrosis and apoptosis. The latter, because it is an active process, is amenable to molecular dissection. Apoptosis is relatively simple to observe in model systems where it is induced using trophic factor withdrawal or chemical treatment. In neurodegenerative diseases, such as Alzheimer’s or Parkinson’s disease, and in stroke, however, the degenerative process is often more protracted, receives contributions from multiple signaling pathways, and the proportion of cell death attributed to apoptosis versus necrosis is less obvious. The contribution that each of these mechanisms makes to the overall cell death process is an expanding area of interest (1, 2). The caspase family of proteases, one of the first gene families implicated in apoptosis, has been studied using a wide range of molecular and enzymatic tools. In particular, a number of tetrapeptide inhibitors have been designed that show selectivity between the caspases and have been used to identify caspase-dependent pathways (3). The processes contributing to neuronal degeneration in a disease such as stroke are highly complex, and it is possible that these inhibitors affect multiple pathways as follows: apoptotic, necrotic, and inflammatory. We have used in vitro neuronal models of oxidative stress and ischemia-induced cell death to study the neuroprotective properties of the tetrapeptide caspase inhibitor Ac-YVAD-cmk† and our own proprietary inhibitors. We observed that the structure activity relationships of the compounds for caspases did not correlate with their neuroprotective efficacy. This strongly suggested that the inhibitors can be effective via a mechanism(s) other than that of caspase inhibition and also that targeting of this alternative mechanism(s) may contribute significantly to the rescue of the neurons from cell death. The identification of alternative mechanisms of neuronal rescue will allow new strategies for intervention in human neurodegenerative disease. In this report we describe the characterization of an alternative target for Ac-YVAD-cmk in neuronal cells by applying affinity labeling methods, previously used to identify activated caspases in apoptosing Jurkat cells (4).

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following suppliers: cell culture supplies were obtained from Life Technologies, Inc., and Tween 20, Ac-YVAD-cmk, bYVAD-cmk, Ac-LV-lysinal, and Z-Phe-Ala-fluoromethyl ketone (Ac-YVAD-cmk) were obtained from Calbiochem-Novabiochem. Tris glycine gels were from NOVEX. Avidin-Neutraltie and biotinylated horseradish peroxidase were from Molecular Probes. Immobilon-P polyvinylidene difluoride membrane was from Millipore Corp. Enhanced chemiluminescence (ECL) reagents and PD10 columns were from Amersham Pharmacia Biotech. Ultralink immobilized neutreavidin plus was obtained from Pierce. Colloidal Gold Total Protein Stain was from Bio-Rad. Sequencing grade trypsin was obtained from Roche Molecular Biochemicals. All other reagents were analytical grade supplied by Sigma unless stated.

Cell Culture and Cell Death Assays—Rat cerebellar cultures

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† The abbreviations used are: Ac-YVAD-cmk, acetyl-Tyr-Val-Ala-Asp chloromethyl ketone; Ac-LV-lysinal, acetyl-Leu-Val-lysinal; bYVAD-cmk, biotinylated-YVAD-cmk; E64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; ECL, enhanced chemiluminescence; MCAO, mid-cerebral artery occlusion; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OGD, oxygen-glucose deprivation; PAGE, polyacrylamide gel electrophoresis; Z-FA-FMK, benzoxycarbonyl-Phe-Ala-fluoromethyl ketone; ANOVA, analysis of variance; DTT, dithiothreitol; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
prepared as described previously (5). Neuronal cultures were prepared from cultures seeded at 24 x 10^4 cells/cm^2 by growing the cultures in basal modified Eagle’s medium (supplemented with 50 mg/liter gentamicin, 10% heat-inactivated fetal calf serum, 25 mM KCl) and incubated at 37 °C in humidified 95% air, 5% CO_2, with the addition on the following day of 10 mM cystine arabinoside to prevent the growth of nonneuronal cells. For astrocyte cell cultures, cerebellar cells were seeded at 9 x 10^4 cells/cm^2 and cultured without the supplemental 25 mM KCl and cystine arabinoside and grown to confluence for two passages. Hippocampal slice cultures were prepared as described (6). Cultures were used after 9–12 days in culture and were preexposed to compounds for 1 h prior to oxygen/glucose deprivation (OGD). OGD was carried out for 35 min, and upon completion the medium was replaced with prewarmed 95% air, 5% CO_2-saturated serum-free medium, containing 6 μg/ml propidium iodide (Molecular Probes, Leiden, The Netherlands) and inhibitors. Viability of cultures was assessed 24 h later by measuring propidium iodide fluorescence in the CA1 region of the hippocampus according to Newell et al. (7). Statistical significance was assessed by ANOVA.

Mouse HT4 neuronal cells were maintained as described (8). Cell death was induced in HT4 cells as described (9). Cells were incubated with 5 mM glutamate for 6 h for a bYVAD binding end point or overnight for cell death end point. Ac-YVAD-cmk was added to the cells just prior to the addition of glutamate. Cell viability was measured at the end of 24 h of exposure to glutamate by observing cellular morphology and assaying the ability to reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) according to the method of Hansen et al. (10).

**Labeling and Identification of YVAD-binding Protein**—Labeling of caspases and affinity blotting were performed as described previously (4), except that protease inhibitors (2 μM each chymostatin, pepstatin, leupeptin, antipain) were omitted from KPM buffer (50 mM KCl, 50 mM PIPES, 10 mM EGTA, 1.92 mM MgCl_2, pH 7, 1 mM dithiothreitol (DTT), 10 μg/ml cytochalasin B) and MDB buffer (50 mM NaCl, 2 mM MgCl_2, 5 mM EGTA, 10 mM HEPES, 1 mM DTT, pH 7), in order to obviate competition for binding between bYVAD-cmk (2 μM) and biotin-labeled proteins. Lysate proteins were labeled with 2 μM bYVAD-cmk, and biotin-labeled proteins were visualized as described under “Experimental Procedures.” B, Jurkat cell lysates were prepared from cells untreated (A and B) or exposed to etoposide (C and D) for 1 h. Lysate proteins were labeled with biotinylated or unbiotinylated “cold” bYVAD-cmk and visualized as described.

**Fig. 1.** Ac-YVAD-cmk protects HT-4 cells from cell death in response to glutamate. HT-4 cells were exposed to vehicle (A) or to 5 mM glutamate for 24 h in the absence (B) or presence (C) of 10 μM Ac-YVAD-cmk. D, the concentration dependence of rescue by Ac-YVAD-cmk. Cells were incubated with a dose response to Ac-YVAD-cmk concurrently with exposure to either vehicle (○) or 5 mM glutamate (●) for 24 h. Cell death was quantitated by staining viable cells with MTT. n = 3, error bars = S.D. Data shown are typical results from three similar experiments. E, rescue of hippocampal slices from OGD-induced damage. Hippocampal slices were preexposed to Ac-YVAD-cmk for 60 min prior to exposure to 35 min of oxygen/glucose deprivation. After 24 h cell death in the CA1 was quantitated by imaging propidium iodide staining. Error bars = S.D.; *, p < 0.005 versus control by ANOVA; data are typical results from three separate experiments.
Subcellular Fractionation of the YVAD-binding Protein—The procedure was adapted from Ref. 11. Primary cerebellar astrocyte cells were resuspended in 2 ml of a 10% sucrose solution, lysed by sonication, and a post-nuclear supernatant obtained by centrifugation at 1500 g for 10 min, the supernatant loaded onto a step sucrose gradient (1.5 ml 50%, 2.5 ml 42%, 2.5 ml 36%, 2.5 ml 32%, 1.3 ml 20% (w/v)). After centrifugation at 66,000 g for 90 min, the particulate fractions were diluted to 12 ml in MDB buffer, pelleted at 137,000 g for 1 h, resuspended in MDB buffer, and labeled as described with 20 μM bYVAD-cmk. Hexosaminidase activity was determined as described previously (12).

Affinity Purification of YVAD-binding Protein—Confluent cerebellar astrocytes were harvested in KPM buffer, lysed, and labeled with bYVAD-cmk as described above, then diluted to 2.5 ml with MDB buffer, and buffer exchanged twice into MDB buffer over two PD10 columns to remove free bYVAD-cmk. The YVAD-binding protein complexes were extracted using two rounds of immunoprecipitation with 300 μl of Ultralink immobilized neutravidin for 1 h. The pooled resin was washed three times with 2 volumes of MDB buffer and eluted with SDS-PAGE reducing buffer (4% (w/v) SDS, 125 mM Tris-HCl, pH 6.8, 100 mM DTT, 20% (w/v) sucrose) for 5 min at 100 °C. Prior to two-dimensional PAGE proteins were precipitated with trichloroacetic acid according to the method of Gullick (13).

Separation of Proteins by Two-dimensional Polyacrylamide Gel Electrophoresis—The protein pellet was dissolved in 375 μl of sample buffer, (2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 0.5% (v/v) DTT, IPG buffer, pH 4–7 (Amersham Pharmacia Biotech)) in a sonicating water bath. Samples were subjected to two-dimensional PAGE using 18 cm, pH 4–7, linear immobilized pH gradient Drystrips (Amersham Pharmacia Biotech) in the first dimension and 24 × 20 cm 12% (w/v) Duracryl-SDS gels (Genomic Solutions) essentially as described by Görg et al. (14).

Co-registration of the bYVAD-bound protein spots on Coomassie gels with those detected on blots was achieved using non-radioactive double detection method of Chevallet et al. (16). Biotinylated proteins were visualized as described earlier (4).
Evidence for Cathepsin-B as a Target for Neuroprotection

RESULTS

Ac-YVAD-Cmk Is Neuroprotective for Neuronal Cell Line HT-4 and Hippocampal Slices—In order to study signaling pathways that may be involved in stroke, we have analyzed neuronal death occurring in response to oxidative stress and ischemic insult. For the former, HT-4 cells were exposed to high concentrations of glutamate over a prolonged time course, a model system that has been shown previously to produce oxidative stress resulting in cell death and that also operates in primary cortical and cerebellar granule cells (9). After 24 h of exposure the number of viable cells was assessed morphologically (Fig. 1A–C) and quantitatively by measuring the reduction of MTT by the remaining viable cells. The cells were rescued from cell death by co-administration of Ac-YVAD-cmk (Fig. 1D) with an EC_{50} of 5 μM. To provide a model more similar to the ischemic conditions thought to occur in stroke, hippocampal slice cultures were subjected to oxygen/glucose deprivation for 45 min and serum withdrawal in the presence of vehicle or Ac-YVAD-cmk. The extent of CA1 damage was assessed after 24 h by propidium iodide staining and clearly showed a protective effect of Ac-YVAD-cmk (Fig. 1E). Induction of caspase activity, however, could not be detected in extracts of the hippocampal slices. Also, caspase inhibitors with >100-fold lower affinity for purified caspases proved to be equipotent inhibitors of cell death in the cellular models (data not shown). As a result we decided to investigate alternative targets for Ac-YVAD-cmk to explain these findings.

Identification of a YVAD-binding Protein—Affinity labeling of cell extracts was performed to identify proteins that bind Ac-YVAD-cmk. HT-4 cells were treated with glutamate, or Jurkat cells were treated with etoposide, and harvested with or without protease inhibitors. The extracts were labeled with 20 μM bYVAD-cmk for 0–30 min. Labeled proteins were then resolved by one-dimensional SDS-PAGE and visualized as described.

Sample Preparation and Mass Spectrometry—Spots corresponding to the bYVAD/ECL signals were excised from Coomassie Blue-stained gel, and in situ trypsin digestion was carried out essentially as described by Shevchenko et al. (17). Prior to analysis by mass spectrometry (MS) all digests were purified by reverse phase chromatography on C18 ZipTips (Millipore, Watford, UK) following manufacturer’s instructions with slight modifications. The bed volume of the resin of 0.6 ml was reduced by approximately half by cutting of a small piece from the front of the tip. Peptides were eluted in 1–2 ml of water/methanol (1:1) containing 5% formic acid. The eluate was immediately loaded into a nanospray microcapillary (type “N,” Protana, Odense, Denmark). Nanospray MS and MS/MS experiments were performed on an orthogonal acceleration quadrupole-time-of-flight mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray ion source for nanospray analysis. Sequence tag searches were performed using PepSea software (Protana, Odense, Denmark) that was installed in-house. Sequence tags were searched against an up-to-date, non-redundant protein data base available from the NCBI.

FIG. 6. Affinity purification and resolution of YVAD-BP by two-dimensional PAGE. Cell lysates were prepared from 17,000 cm² of confluent cerebellar astrocytes and labeled with bYVAD-cmk, and binding proteins were affinity-purified and resolved by two-dimensional PAGE. A, a, Coomassie-stained gel; B, b, combined images of colloidal gold-stained blot and avidin/biotin-horseradish peroxidase/ECL signal. a and b are expansions of A and B; circles highlight co-registered spots subjected to sequencing.

FIG. 7. Competition for YVAD-BP by cathepsin-B inhibitor. Lysates were prepared in the presence of 2, 20, or 200 nM Ac-LV-lysinal and then labeled with 2 μM bYVAD-cmk for 0–30 min. Labeled proteins were then resolved by one-dimensional SDS-PAGE and visualized as described.
only labeling of a single band at 32 kDa (data not shown), suggesting that this band is the predominant target for bYVAD-cmk. In contrast, affinity labeling of Jurkat extracts with bYVAD-cmk revealed the expected labeled proteins at 17–24 kDa in etoposide-treated cells. These bands correspond to activated caspase-3 (4), but a major band at 32 kDa was absent (Fig. 2B).

**YVAD-binding Protein Is Targeted during Rescue with YVAD-Cmk**—The YVAD-BP should be targeted by Ac-YVAD-cmk during the neuroprotection assay if its inhibition is important to cell rescue. To test this, lysates were prepared from naïve HT-4 cells or cells that had been rescued from glutamate-induced death by co-administration of Ac-YVAD-cmk (20 μM). The cells were washed, harvested, and lysed, and extracts were subjected to labeling with bYVAD-cmk and products run out on one-dimensional SDS-PAGE, as described. Compared with extracts from the control naïve cells, labeling of the 32-kDa band was inhibited in extracts from the cells that had been rescued from cell death by Ac-YVAD-cmk (Fig. 3A), indicating that the YVAD-BP is a target protein for the inhibitor during cell rescue.

The 32-kDa YVAD-BP Targeted by bYVAD-Cmk Is a Cysteine Protease—The labeling of the 32-kDa band had been initially revealed by extracting in the absence of known protease inhibitors, suggesting that the 32-kDa YVAD-BP is a protease and that a class-specific protease inhibitor inhibits the binding of bYVAD-cmk. Hence, different protease inhibitors, chymostatin, pepstatin, leupeptin, antipain, E64, and phenylmethylsulfonyl fluoride, were added to the extraction buffer before affinity labeling with bYVAD-cmk and analysis by SDS-PAGE. In order to obtain YVAD-BP more efficiently, and avoid background from co-migrating bands, alternative cell types were screened for the YVAD-BP (Fig. 3B). Subsequently, cerebellar granule cells were used for competition studies and astrocytes for purification purposes. Leupeptin, antipain, and E64 inhibited the labeling of bYVAD-cmk, indicating that the YVAD-binding protein is likely to be a cysteine protease (Fig. 4). Cysteine proteases include the caspases commonly associated with cell death pathways and also cysteine cathepsins such as cathepsins-B or -L. Because caspases are usually activated during the induction of cell death, the data showing constitutive activity in control cells weighed against the YVAD-BP being a caspase-like activity (Fig. 2A).

**Subcellular Fractionation of the YVAD-binding Protein**—The 32-kDa YVAD-BP might be expected to be enriched in the lysosomal fraction of cells if it is a cysteine cathepsin. Subcellular fractions were separated on sucrose gradients and labeled with bYVAD-cmk. The fractions were also assayed, in parallel, for the specific lysosomal protein hexosaminidase. The YVAD-BP was found in the cytoplasm and the lysosomal fractions 4 and 5, co-fractionating with hexosaminidase (Fig. 5). This suggests that the 32-kDa YVAD-BP is enriched in lysosomes, which is consistent with it being a cathepsin.

**Affinity Purification of YVAD-binding Protein**—The bYVAD-cmk-labeled extract was buffer-exchanged to remove unbound bYVAD-cmk, then affinity-purified using Neutravidin beads, and the eluted proteins resolved by two-dimensional PAGE as described under “Experimental Procedures.” The available protein was run out on two-dimensional PAGE in two batches. A larger loading (9/10 sample) gel was stained with Coomassie Brilliant Blue R-250 (Fig. 6A, a). A second (1/10 sample) gel was used to co-register protein spots with ECL signals, using a blot probed with both colloidal gold and avidin/ECL for the labeled proteins. The ECL and gold images were overlaid, and labeled proteins were identified (Fig. 6B, b). Comparison of ECL and protein-stained images allowed cross-identification of spots on the Coomassie gel.

Three spots of approximately the same molecular weight were detected by ECL with a signal saturating the film. Parallel spots were detected using protein stains and showed clear differences in protein levels (Fig. 6). The Coomassie spots outlined were excised from the gel and prepared for and subjected to electrospray ionization mass spectrometry.

**Sequencing of YVAD-BP by Mass Spectrometry**—Nanospray-MS analysis of the digests revealed one peptide at m/z 996, among others from keratins, that was observed in the digests of all the target spots but not in a control digest of an unstained region of the gel. The peptide was subjected to MS/MS analysis, and a sequence tag (18) of VTFAGEV was deduced from the MS/MS spectrum. A sequence tag search against a non-redundant protein data base containing ~340,000 entries revealed a single hit to cathepsin-B (rat). This clearly identifies the YVAD-BP present in the analyzed bands as cathepsin-B.

**Competition for YVAD-BP between bYVAD-Cmk and Cathepsin-B Inhibitors**—The recognized cathepsin-B inhibitors...
Ac-LV-lysinal and Z-FA-fmk were tested for their ability to compete with 2 μM bYVAD-cmk for the YVAD-BP in cell extracts. Cell extracts were prepared in the presence of inhibitor and then incubated with bYVAD-cmk, an aliquot being taken and stopped in SDS-PAGE sample buffer at 0–30-min time intervals. The labeled YVAD-BP was visualized as described previously. Both Ac-LV-lysinal (Fig. 7) and Z-FA-fmk (data not shown) successfully competed for the binding protein at a concentration between 20 and 200 nM, supporting the sequencing shown) successfully competed for the binding protein at a concentration between 20 and 200 nM, supporting the sequencing data showing the 32-kDa band to be cathepsin-B.

Neuroprotective Property of Cathepsin-B Inhibitor Z-FA-Fmk—Because the target protein was identified as cathepsin-B, one would expect cell-permeable inhibitors of cathepsin-B to be able to rescue neuronal cultures. Propertiy and the commercially available cathepsin-B inhibitor Z-FA-fmk were, therefore, tested for a neuroprotective effect in the hippocampal slice culture model of ischemia, in which Ac-YVAD-cmk is protective. Hippocampal slices were exposed to OGD with or without the cathepsin-B inhibitor Z-FA-fmk. At 24 h clear OGD-induced damage was measured in control cultures. Cultures treated with inhibitor, however, showed a significant (p < 0.005 ANOVA) reduction of ~40% in damage, compared with damage in cultures subjected to OGD in the absence of the cathepsin-B inhibitor (Fig. 8). Alternative cathepsin-B inhibitors to Ac-YVAD-cmk, therefore, are able to rescue the hippocampal slice culture from ischemic stress and, hence, support the evidence that cathepsin-B contributes to the cell death occurring in this model of neurotoxicity.

DISCUSSION

In our study we have traced a path starting from our primary observations of the neuroprotective properties of one of the caspase tetrapeptide inhibitors, ac-YVAD-cmk, not to the expected caspase but to cathepsin-B. The tetrapeptide caspase inhibitors have been shown to be neuroprotective in a wide range of different neuronal models (19). The caspase-3 inhibitors based on DEVD inhibit apoptosis in neuronal cultures subjected to neurotrophic factor withdrawal, potassium withdrawal, or chemical induction. YVAD-based inhibitors have been shown to be protective in fewer in vitro models but, perhaps more importantly, have been shown to reduce lesion volume in MCAO models of stroke (20). Given the efficacy of YVAD inhibitors in MCAO models, a suggestion that an alternative target might be involved highlights an opportunity for furthering the understanding of the pertinent mechanisms in cell death. Our data suggest that the inhibition of cathepsin-B by Ac-YVAD-cmk may contribute to its neuroprotective effect. The data also show a primary demonstration of a rescuing effect of the inhibition of cathepsin-B in neuronal cultures exposed to oxidative stress or modeled ischemia. The results also caution against the interpretation of data obtained with micromolar concentrations of Ac-YVAD-cmk solely in terms of caspase inhibition.

Evidence for and against a role for cathepsin-B in promoting cell death is present in the literature. On one hand, cathepsin-B has been reported to be a downstream effector protease responsible for apoptosis in hepatocytes, following exposure to bile salts (12) or TNF-α (21). In contrast, cathepsin-B appears to be neuroprotective in neuronal PC12 cells undergoing apoptosis in response to serum withdrawal (22). Inhibition of cathepsin-B caused death, which could be blocked by inhibition of the aspartyl cathepsin-D (22). This work suggests cathepsin-B protects from the effects of cathepsin-D and concurs with the identification of cathepsin-D as a proapoptotic gene identified by expression cloning (23). Although the PC12 data suggest that cathepsin-B activity is neuroprotective in neuronal cells, data from in vivo studies suggest the opposite. Cathepsin-B levels are reported to increase post-ischemia and to relocate from the lysosomes to the cytoplasm (24, 25). Furthermore, administration of cysteine protease inhibitors has been reported to reduce cerebral infarct volumes and cell death in the CA1 region of the hippocampus in particular (26, 27). Although the in vitro effect of the cathepsin inhibitors is striking, attributing the effect to action at a particular enzyme needs to be viewed with the same caution as for the caspase inhibitors. However, the in vitro culture models employed in this study, particularly the slice culture exposed to oxygen-glucose deprivation, have been chosen on account of their similarity to stroke in vivo, and in contrast to the PC12 cell data, our results support a role for cathepsin-B in neuronal cell death. Many signaling and trophic mechanisms are likely to impact upon cell survival in stroke, and Ac-YVAD-cmk may be effective on account of actions at several of these. Coincidentally, while studying the role of YVAD in the inhibition of the interleukin processing cascade in liver cells, Schotte et al. (12) observed that Ac-YVAD-cmk is able to inhibit cathepsin B. Several reports attest to the effectiveness of YVAD in the MCAO model of stroke in terms of inhibition of formation of interleukin-1 and reduction of lesion volume (20). The results presented here suggest that inhibition of cathepsin-B may make a further contribution to these protective properties and highlights the possibility of employing cathepsin inhibitors for the treatment of stroke or other neurodegenerative diseases, an approach that is gaining increasing attention (28, 29).

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*J. Biol. Chem.* 2001, 276:32750-32755.
doi: 10.1074/jbc.M103150200 originally published online June 26, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103150200

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