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Okadaic Acid Induces Cycloheximide and Caspase Sensitive Apoptosis in Immature Neurons

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Previous studies have shown that okadaic acid (OA) evokes tau phosphorylation and neurofibrillary changes *in vivo*, and in cultured neurons, that resemble Alzheimer's disease pathogenesis. In order to investigate the mechanism of OA-neurotoxicity, we treated cultured rat neurons with OA and examined nuclear morphology, phosphatidylserine (PS) externalization, α-fodrin cleavage, and the effects of cell death inhibitors. Our results demonstrated that cycloheximide (CHX) and the broad-spectrum caspase inhibitor, ZVAD, significantly reduced cell death in a dose-dependent manner. Nuclear fragmentation, a hallmark of apoptosis, occurred after OA treatment and was inhibited by CHX or ZVAD. PS externalization was apparent in 6–12 h in neurites and in cell bodies, and peaked at 24 h after OA treatment. Cleavage of α-fodrin as visualized by the appearance of 150- and 120-kDa bands appeared with a time course similar to PS externalization. These results suggest that OA induce CHX and caspase sensitive neuronal apoptosis.

Keywords: Apoptosis; Caspase; Cycloheximide; Okadaic Acid.

Introduction

Apoptotic cell death is a fundamental biological process critical for the development of the organism and the maintenance of tissue homeostasis. In particular, development of the CNS includes restricted periods of naturally occurring cell death. Cells undergoing apoptosis are characterized morphologically by plasma membrane blebbing, cell body shrinkage, and chromatin condensation. The transduction of apoptosis requires the coordinated activation of several caspases, a class of aspartate-specific cysteine protease, and other proteases (Cohen, 1997; Nicholson and Thornberry, 1997; Pettmann and Henderson, 1998; Vanags *et al.*, 1996). The current definition of apoptotic pathways identifies specific markers of apoptosis, which include cleavage of various targets of activated caspases and exposure of phosphatidylserine (PS) on the cell surface (Castedo *et al*., 1996; Holtsberg *et al*., 1998). Externalization of PS, a lipid normally confined to the inner leaflet, has been shown to occur during apoptosis induced by multiple apoptotic stimuli, including trophic factor withdrawal, fas ligand, and staurosporine in many types of cells (Martin *et al*., 1995; Rimon *et al*., 1997). Externalization of PS during apoptosis provides a signal for phagocytic cell recognition, preventing secondary necrosis and inflammation (Fadok *et al*., 1992). Proteolysis of α-fodrin has been reported as another important change during apoptosis that can be induced by a variety of stimuli (Cryns *et al*., 1996; Martin *et al*., 1995; Vanags *et al*., 1996). Fodrin is a component of the neuronal cytoskeleton that is responsible for coupling membrane-spanning cell surface proteins to cytoplasmic elements, and is also associated with the maintenance of lipid symmetry by anchoring PS at the cytofacial membrane (Bennett and Gilligan, 1993). Cleavage of the 240-kDa α-fodrin subunit into 150- and 120-kDa products has been attributed to the involvement of calpain or caspases. Thus, it has been speculated that fodrin proteolysis during apoptosis might contribute to membrane-associated morphological features of apoptosis, e.g. membrane blebbing, cytoplasmic shrinkage, and the rounding up of cells (Bennett and Gilligan, 1993) .

A profound loss of neurons is an invariant feature of Alzheimer's disease (AD) and it is correlated with

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Abbreviations: AD, Alzheimer's disease; CHX, cycloheximide; LDH, lactate dehydrogenase; OA, okadaic acid; PS, phosphatidylserine; ZVAD, *N* benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone.

neurofibrillary tangles (Gomez-Isla *et al*., 1997; 1996; Hyman *et al*., 1990; 1984). Recently, neuronal loss by apoptosis has been reported to occur in AD as well as in other neurodegenerative diseases (Anderson *et al*., 1996; Cotman and Su, 1996; Su *et al*., 1994). Activated microglia are closely associated with neurons and processes that are positive for C1q complement in the AD brain, and these microglia may be involved in the apoptotic removal of degenerating neurites (Afagh *et al*., 1996). Previous studies have suggested that okadaic acid (OA) induces hyperphosphorylation of tau in cultures (Arias *et al*., 1993; Malchiodi-Albedi *et al*., 1997; Vandermeeren *et al*., 1993). *In vivo*, it leads to the deposition of Aβ positive plaques, subsequent neuronal degeneration, loss of synapses, memory impairments, all of which resemble AD-like pathology (Arendt *et al*., 1995). Our recent study has also shown that OA-induced neurofibrillary pathology in primary cultures replicates many aspects of AD pathology (Kim *et al*., 1999). In the present study, we have examined the possibility that OA can initiate apoptosis in cultured hippocampal neurons by evaluating nuclear chromatin fragmentation and extranuclear apoptotic events such as PS exposure, fodrin proteolysis, and the effects of caspase inhibitors on neuronal apoptosis.

Materials and Methods

Cell culture Embryonic cultures of hippocampal neurons were prepared from Sprague-Dawley rats at gestational day 18 as described previously (Pike *et al*., 1993). Briefly, hippocampi were removed and dissociated with 0.125% trypsin in calcium- and magnesium-free buffered HBSS for 10 min at 37°C. Trypsin was inactivated with Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 10% fetal bovine serum before dissociation of cells by titration using a Pasteur pipette. The neurons were plated at 5×10^5 cells/well on poly-L-lysine-treated 12-well plates, and were maintained in DMEM media supplemented with N_2 components (Brewer *et al*., 1989). The neurons were kept in culture at 37°C with 5% CO_2 for 3–4 d prior to the addition of OA.

Cell death and viability assays Neuronal cells were exposed to 20 nM OA after 1 h pretreatment of vehicle or inhibitors. Okadaic acid (Boehringer Mannheim, 1 μM) was dissolved in 0.1% DMSO. ZVAD or DEVD-fmk (Enzyme system products, 50 mM) and YVAD (Enzyme system products, 12.5 mM) were dissolved in DMSO, and cycloheximide (CHX) was dissolved in water (100 μg/ml). Cells were photographed under phase-contrast microscopy and quantified by lactate dehydrogenase (LDH) assay using the method described previously (Koh and Choi, 1987). The final levels of LDH released from control and treated cells varied from plate to plate due to the variability in cell density. Therefore, in order to compare one plate to another, all values were normalized to cultures treated with calcium ionophore (1 μM, A23187), which defined the maximal LDH release (assigned 100%). In DEVD-fmk experiments, cell survival was evaluated by colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), because the half-life of DEVD-fmk is relatively short and the LDH assay cannot reflect early cell damage. MTT reduction was expressed as the ratio of the signal obtained from inhibitor-treated cultures to the signal from vehicle-treated control cultures multiplied by 100 (percentage control).

Assessment of nuclear morphology Nuclear morphology was monitored with the membrane permeable dye SYTO 11 Live Cell Nucleic Stain (Molecular Probes, Eugene, OR). Briefly, neurons were exposed to 1 μM SYTO 11 and 2 μM calcein AM (Molecular Probes, Eugene, OR) in Dulbecco-PBS for 10 min at 37°C and were observed for nuclear condensation and fragmentation, characteristics of cells undergoing apoptosis, using fluorescence microscopy.

Phosphatidylserine exposure PS exposure was assessed for binding of annexin V according to a previous method with modifications (Koopman *et al*., 1994). Neurons were rinsed with HEPES buffer (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$), then incubated with biotinylated annexin V (CalTag, 5 μg/ml) in HEPES buffer for 30 min at 37°C. Following this incubation, the cells were quickly washed 3 times with HEPES buffer, then fixed by underlay with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min. Cells were washed in PBS, blocked with 2% horse serum/2% BSA in PBS, and incubated with avidin-HRP complex (1:300, Vector Labs, CA). The final color product for labeling was obtained using 0.05% diaminobenzidine with 0.01% H_2O_2.

Western blot analysis Cultures were solubilized directly in 200 μl of sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 2.5% glycerol, 0.5% 2-β-mercaptoethanol, and bromphenol blue), boiled at 100°C for 5 min, and stored at −20°C until use. Equal amounts of sample were subjected to SDS-PAGE (12% acrylamide) at constant voltage (130 V) and were subsequently transferred to polyvinylidene difluoride membrane (pore size, 0.2 μm, Biorad) at 110 V for 2 h. After 1 h incubation in blocking TTBS buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween-20) containing 2% BSA with 2% NHS, the blots were incubated with anti-mammalian brain α-fodrin polyclonal antibody (1:125, AB992, Chemicon international) for 1 h at RT. The blots were washed in TTBS buffer and subsequently incubated with HRP-labeled anti-rabbit IgG (Pierce, 1:10000) solution for 1 h at RT, and visualized using enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL) and X-ray film.

Results

Treatment of neurons with OA resulted in apoptotic neuronal death Most of the neurons showed neurite retraction within 2–12 h of OA treatment, after which they progressively underwent a profound loss of neurites, rounding of the soma, and detachment from the substratum (Fig. 1B). A membrane-permeable SYTO 11 nucleic acid stain was used to monitor changes in nuclear morphology that occurred as a result of exposure to OA. Vehicle- and CHX-treated neurons exhibited round, smooth, and large nuclei, and displayed uniform staining (Figs. 1D and 1F).

In contrast, after OA treatment, the nuclei appeared to be condensed, fragmented, and not uniform, although they were still calcein AM positive (Fig. 1E, arrow). These changes in nuclear morphology were similar to those observed when cells were treated with concanavalin A or staurosporine, a protein kinase inhibitor reported to induce apoptosis in many types of cells in culture (Ahn *et al*., 1999; Cribbs *et al*., 1996; Jarvis *et al*., 1994; Raff *et al*., 1993). Although neurons protected with CHX and ZVAD were morphologically distinguishable from control neurons by damaged short neuritic process and rounding of cell bodies (Fig. 1C), these results provide evidence that the OA-induced neuronal death was attributable to apoptosis. OA treatment induced more than 50% neuronal death after 40 h, as assessed by LDH release (Fig. 2). OA-induced neuronal death was inhibited with either 1 μg/ml CHX or ZVAD pretreatment in a dose-dependent manner (Fig. 2). The inhibitory effect of CHX was also dose-dependent (data not shown).

Externalization of phosphatidylserine In these assays, biotinylated annexin V was used to detect PS exposure on cells. Extracellular PS exposure is indicated by a positive annexin V signal in cells with intact plasma membranes. PS exposure first appeared on neurites at 6 h and subsequently cell bodies at 12 h, and was maximal at 24 h after OA treatment (Fig. 3). These results are consistent with previous data that showed that the appearance of PS

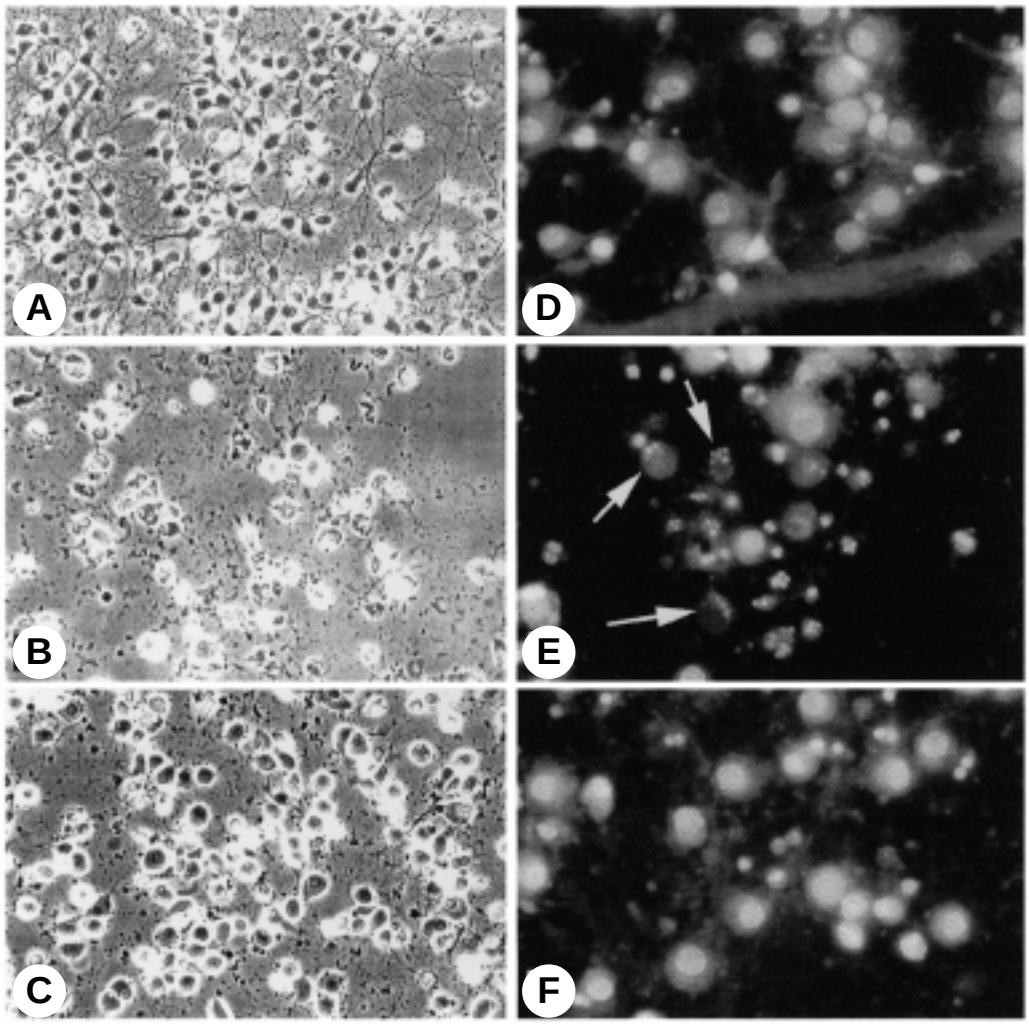

Fig. 1. OA evokes apoptosis of neuronal cells. Cultures at 4 d *in vitro* (DIV) were treated with vehicle (**A**, **D**), OA (20 nM; **B**, **E**), and OA plus 1 mg/ml cycloheximide (**C**, **F**) for 40 h. Phase-contrast views are shown in A, B, and C (200×). Nuclear changes were shown using the membrane-permeable nucleic acid stain SYTO 11 and neuron viability was shown using calcein AM in **D**, **E**, **F** (600×). Control neurons showed normal morphology (**A**), and calcein AM-positive cytoplasm, including large bundles of neurites (**D**). OA treatment induced marked neuronal death and cell detachment (**B**); condensed and fragmented nuclei in OA treated neurons that are still calcein AM-positive (**E**, arrows). OA-induced neuronal death and nuclear changes were inhibited by cycloheximide (1 μg/ml, **C**, **F**).

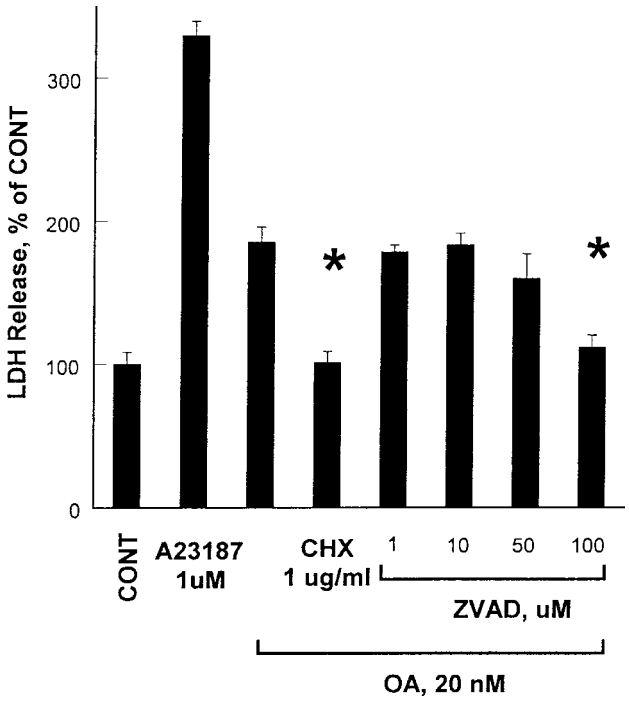

Fig. 2. Effect of the inhibition of protein synthesis (1 μg/ml, cycloheximide) and the inhibition of caspase activity (ZVAD) on cell viability in cultures (4 DIV) treated with OA (20 nM) for 40 h. Neuronal death was evaluated by the release of lactate dehydrogenase (LDH). Values represent LDH release (mean + SEM, n = 8) and are expressed as a percentage of LDH release of control. ($P < 0.05$, Anova with Fisher correction)

in the outer-leaflet of the extracellular membrane is an early and pervasive feature of apoptotic cells (Rimon *et al*., 1997; Vanags *et al*., 1996).

Selective cleavage of α-fodrin during OA-induced neuronal apoptosis: differential inhibition by CHX and ZVAD Cleavage of α-fodrin was evaluated in cultures treated with okadaic acid (OA). As shown in Fig. 4A, a band of 240 kDa, corresponding to intact α-fodrin, was the major band present in control cultures. The level of intact α-fodrin decreased significantly after OA treatment with the appearance of cleavage products, yielding major detectable fragments of 150 kDa and 120 kDa. In contrast, only the 150-kDa product appeared after A23187 treatment. ZVAD inhibited the production of the 120-kDa fragment following OA treatment, suggesting that the band at 120 kDa is specific for caspase activation during apoptosis. The α-fodrin cleavage into the 120-kDa band during OA-induced apoptosis was largely inhibited by preincubation with CHX and ZVAD, while the 150-kDa band was strongly inhibited only by CHX (Fig. 4A).

Next, we examined the temporal changes on α-fodrin proteolysis after OA treatment (Fig. 4B). A time dependent decrease of intact α-fodrin (240 kDa) was observed concomitant with the generation of 150-kDa and 120-kDa breakdown products. Initial cleavage products were detectable about 6 h after the addition of OA, with an almost complete loss of α-fodrin seen by 36 h. This temporal change in α-fodrin degradation had a similar time course to PS exposure in OA-treated neurons (Fig. 3), suggesting that the PS exposure could be at least partly maintained by interactions with cytoskeletal fodrin.

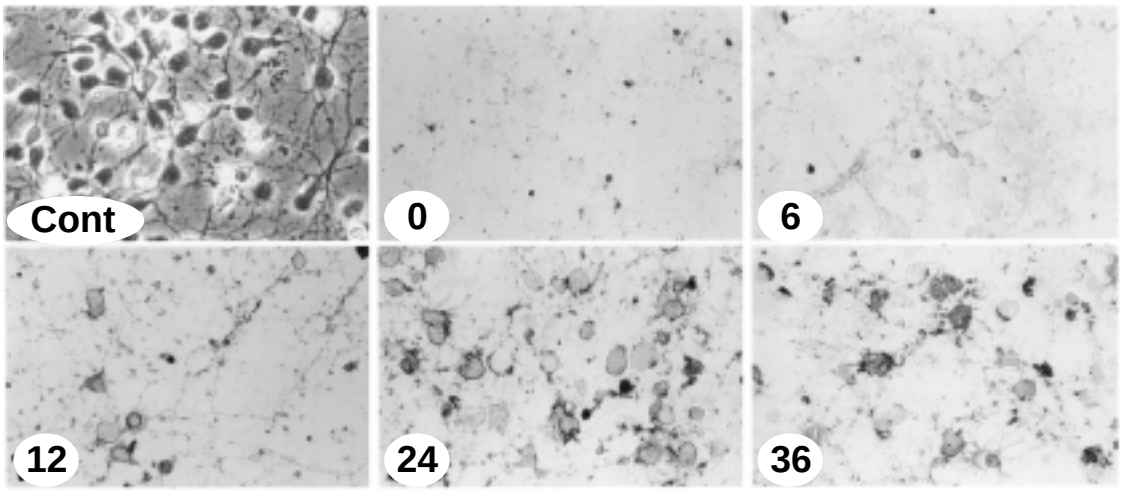

Fig. 3. Binding of annexin V after OA treatment. Neuronal cultures (4 DIV) were incubated with 20 nM OA for the number of hours indicated, and assayed for phosphatidylserine exposure. Annexin V binding in some neurites was detectable by 6 h after OA treatment and the binding at the cell surfaces increased progressively and was maximal at 24 h. (CTL; Control)

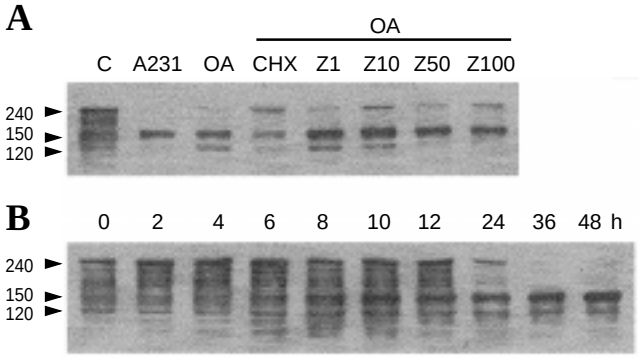

Fig. 4. Cleavage of α-fodrin in cells exposed to okadaic acid and the inhibition of the cleavage by cycloheximide or ZVAD (**A**). Immunoblots, using an axon/presynaptic element-specific anti- α-fodrin antibody was presented. After OA treatment, the level of the 240-kDa, intact α-fodrin was significantly reduced, whereas the content of the breakdown product 150-/120-kDa increased. In contrast, after A23187 treatment, only 150-kDa fragments were increased. Pretreatment with CHX inhibit 150-/120-kDa band production following OA treatment, whereas ZVAD inhibit the 120-kDa band. The temporal changes in α-fodrin cleavage after OA treatment are shown (**B**). C, vehicle treated control; A231, 1 μM A23187 (24 h); OA, 20 nM okadaic acid (40 h); Cycloheximide (1 mg/ml; CHX) or ZVAD was added 1 h before OA treatment.

Discussion

In this study, we have demonstrated that OA-induced neuronal death exhibits the hallmarks of apoptosis, including nuclear fragmentation, PS exposure on the outer leaflet of the plasma membrane, and fodrin proteolysis (Figs. 1, 3, and 4). In addition, neurons rounded up, lost connections with neighboring neurons, and were detached from the substrate. These results are consistent with a recent study demonstrating OA induces apoptosis in a human neuroblastoma cell line (Nuydens *et al*., 1998).

With the identification of several members of the caspase family, it appears likely that distinct caspases may be utilized by different cell types in response to different apoptotic-inducing stimuli (D'Mello *et al*., 1998; Nicholson and Thornberry, 1997; Pettmann and Henderson, 1998). Caspase-3 deficient mice show a number of defects in the development of brain apoptosis (Kuida *et al*., 1996). Strikingly, these defects do not extend to other organ systems. Caspase-3, therefore, seems likely to play an important role in the regulation of cell death in the nervous system (Armstrong *et al*., 1997). Previous studies have shown that activation of caspase-3 either leads to cleavage of DNA fragmentation factor, a cytosolic factor that mediates DNA fragmentation, or to cleavage of ICAD (inhibitor of caspase-activated deoxyribonuclease) to release CAD (caspase-activated deoxyribonuclease) (Enari *et al*., 1998; Liu *et al*., 1997). Our results demonstrate that CHX or ZVAD protects chromatin from nuclear fragmentation in response to OA (Fig. 1). These results support the concept that caspase activation is involved in the apoptotic process and occurs upstream from nuclear fragmentation in the sequence of events leading to cell death (D'Mello *et al*., 1998; Liu *et al*., 1997). Although the protection from OA-induced death by CHX and ZVAD indicated that caspases are activated during apoptosis, the actions of these drugs are relatively broad. ZVAD, an irreversible broad-spectrum caspase inhibitor, inhibits apoptosis induced by a wide range of stimuli in a number of different systems (Cohen, 1997). As a step towards identifying the caspases involved, we examined the effects of a selective caspase-1 inhibitor, YVAD, and the relatively specific caspase-3 inhibitor, DEVD-fmk (Liu *et al*., 1997; Nicholson *et al*., 1995). Our data showed that DEVD-fmk inhibited OA-neurotoxicity in a dose-dependent fashion, however, YVAD did not show any protective effect (data not shown). Recent studies showed that DEVD-fmk, relatively specific to caspase-3, also inhibits several other caspases (caspase-1, -7, -10) (Fernandes-Alnemri *et al*., 1995; 1996; Livingston, 1997). ZVAD inhibits apoptosis and processing of CPP32 (caspase-3), Ich-1 (caspase-2), Mch2 (caspase-6), and Mch3 (caspase-7), which suggests that ZVAD inhibits the most upstream target proteases such as caspase-8 or- 10 (MacFarlane *et al*., 1997). However, the lowest expression of caspase-7, caspase-8, and caspase-10 were reported in the brain (Fernandes-Alnemri *et al*., 1995; 1996). Our results suggest that caspase-3 or other related members, but not caspase-1 itself, may be involved in OA-induced neuronal apoptosis.

Our results also showed that α-fodrin cleavage occurred in OA-induced apoptosis, yielding major detectable fragments of 150-kDa and 120-kDa (Fig. 4). The 120-kDa α-fodrin cleavage band during OA was largely inhibited by preincubation with CHX or ZVAD (Fig. 4). These results are consistent with previous findings showing that caspase-3 (CPP32) produces a 120-kDa fodrin fragment in neuronal apoptosis, but this fragment was not dependent on calpain or caspases-1, -2, or -4 (Nath *et al*., 1996; Vanags *et al*., 1996). CHX prevents caspase-3 activation (Armstrong *et al*., 1997), however, it is interesting to mention that CHX inhibited fodrin proteolysis in not only the 120-kDa fragment but also the 150-kDa fragment. Previous studies suggested that the α-fodrin proteolysis into the 150-kDa fragment during apoptosis may be a consequence of calpain activation (Nath *et al*., 1996; Vanags *et al*., 1996). Therefore, CHX may inhibit calpain-like protease activity, in addition to upstream inhibition of caspase-3. In summary, our results demonstrate that OA triggers morphological and biochemical characteristics of apoptotic neuronal death. CHX and ZVAD inhibited OA-induced neuronal apoptosis.

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