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Apoptotic Morphology Does not Always Require Caspase Activity in Rat Cerebellar Granule Neurons

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The death of a cell via apoptosis is characterized by morphological changes including cell shrinkage and nuclear condensation. Intracellularly, proteases, including caspases, are activated. In the present article we have compared the ability of three different neurotoxic agents to induce caspase activity in cerebellar granule cells (CGC). These compounds are the microtubule-disrupting agent colchicine and the oxidative stress-inducing agents hydrogen peroxide and methylmercury (MeHg). We have previously shown that each of these agents causes nuclear changes that are consistent with apoptosis, i.e., induction of chromatin condensation and DNA cleavage into fragments of regular size (700, 300 and 50 kbp). However, only colchicine causes a large increase in caspase activity, as monitored by the ability of whole cell extracts to cleave the synthetic caspase substrate DEVD-MCA. In contrast, MeHg and hydrogen peroxide do not induce any significant increase of DEVDase activity as compared to control cells. Immunocytochemistry confirms that active caspase-3 is abundant only in colchicine-exposed cells. In agreement with these findings, the pan-caspase inhibitor, z-VAD-fmk, is efficient in protecting CGC against colchicine, but not against hydrogen peroxide or MeHg. These data suggest that in CGC the activation of caspases is not always required to induce morphological changes and pattern of DNA fragmentation consistent with apoptosis.

Keywords: Apoptosis; Caspase-3; Cerebellar granule cells; Colchicine; Hydrogen peroxide; Methylmercury; Oxidative stress

INTRODUCTION

Apoptosis was originally defined by morphological criteria as a cell death process characterized by nuclear condensation, chromatin condensation and margination, cellular shrinkage and fragmentation (Kerr *et al.*, 1972). These changes have long been the defining characteristics of a cell undergoing apoptosis and have served to distinguish this mode of cell death from necrosis, where there is swelling of the cell and its organelles. Since the early 1970s there has been a considerable increase in our understanding of the biochemical processes that underlie these morphological changes. One of the first identified biochemical changes, i.e., progressive regular DNA cleavage into high-molecular weight

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fragments followed by oligonucleosomal laddering, linked to the activation of specific endonucleases, has become an established hallmark of apoptosis (Wyllie, 1980). This laddering is a relatively late event in apoptosis.

More recently, the caspase family of proteases has emerged as having particular importance in achieving the morphological changes that occur during apoptosis in a variety of cell types, including neuronal cells (Gorman *et al.*, 1998). Caspase activation is one of the early steps committing a cell to apoptosis. These enzymes are a family of at least 14 cysteine proteases that specifically cleave after aspartate residues (Thornberry and Lazebnik, 1998). Their long list of substrates includes the DNA repair enzyme, poly(ADP-ribose) polymerase (PARP) and the cytoskeletal protein, fodrin (Stroh and Schulze-Osthoff, 1998).

Caspases are normally present in the cell in an inactive proform, which requires proteolytic processing to generate the heterodimeric active enzyme. Based on their substrate specificities caspases can be subdivided into three groups (Nicholson and Thornberry, 1997). Group III caspases (which include caspases-8, –9 and –10) are the initiator caspases of apoptosis, being largely involved in cleavage of group II caspases resulting in activation of the latter. Group II caspases are sometimes referred to as the executioner caspases since they are largely responsible for cleaving the many proteins that are degraded during apoptosis and they include caspases-2, –3 and –7. Group I caspases are involved in cytokine production and are not directly linked to apoptosis.

Primary cultures of rat cerebellar granule neurons (CGC) are a useful experimental model to study mechanisms of neuronal cell death. The cells are dissociated from the cerebellum within the first postnatal week, when their differentiation is not yet completed, and seeded *in vitro*. After one week the culture consists of a highly homogenous population of differentiated CGC. These cells are known to be sensitive to a wide variety of treatments that mimic the conditions that occur in neurodegenerative disorders. For example, they are sensitive to the toxic effects of colchicine (Bonfoco *et al.*, 1995; Gorman *et al.*, 1999), which has been used to induce cytoskeletal damage similar to that occurring in Alzheimer's disease (Nakagawa *et al.*, 1987; Mattson, 1992). These cells are also sensitive to oxidative stress (Ahlbom *et al.*, 1999; Götz *et al.*, 1999), which is implicated in many neurodegenerative disorders (Gorman *et al.*, 1996; Sun and Chen, 1998) including Alzheimer's disease (Behl, 1999), Parkinson's disease (Jenner and Olanow, 1996) and amyotrophic lateral sclerosis (Cookson and Shaw, 1999). They have also proved to be useful models to study the neurotoxic mechanisms of environmental toxins, such as methylmercury (MeHg) (Kunimoto, 1994; Castoldi *et al.*, 2000; Daré *et al.*, 2000).

The present article summarizes and extends the work that we have done in the past few years aimed at clarifying the relation between induction of apoptotic morphology, DNA fragmentation and caspase activation in CGC exposed to colchicine, hydrogen peroxide and MeHg. Previous reports (see Bonfoco *et al.*, 1995; Gorman *et al.*, 1999; Ahlbom *et al.*, 1999; Gotz *et al.*, 1999; Daré *et al.*, 2000) concluding that these treatments induce apoptosis were primarily based on the altered morphology of the cells, in particular the observation of chromatin condensation, and on the finding of DNA fragmentation into high-molecular weight fragments of $\geq$ 50 kbp. Therefore, we have extended this work to determine the relationship between these nuclear changes and caspase activation in CGC exposed to the three toxic agents.

METHODS

Experimental Animals

Pregnant Sprague-Dawley rats (B&K, Stockholm, Sweden) were housed singly and checked

twice daily. Animals were kept in air-conditioned quarters, with a controlled photoperiod (14 h light: 10 h darkness) and free access to food and tap water. Procedures used in animal experimentation comply with the Karolinska Institute's regulations for the care and use of laboratory animals.

Cell Culture and Treatments

Cerebellar granule cells (CGC) were prepared from pups on postnatal day 7 as previously described (Schousboe *et al.*, 1989; Ahlbom *et al.*, 1999; Gorman *et al.*, 1999). In brief, the cerebella from rat pups were dissected, minced with a McIlwain tissue chopper (Histo-Lab, Gothenburg, Sweden), dissociated with trypsin (Life Technologies, Gibco BRL, Grand Island, NY, USA) and seeded on dishes (or glass coverslips for microscopic analysis) coated with poly-L-lysine (Sigma, St. Louis, MO, USA) (MW 300,000), at a density of 500,000 cells/cm^2. Cells were maintained in basal Eagle's medium (Life Technologies, Gibco BRL, Grand Island, NY, USA) supplemented with 10% inactivated FCS (fetal calf serum)(Sigma, St.Louis, MO, USA), 25 mM potassium chloride and 0.5% (v/v) penicillin-streptomycin (Life Technologies, Gibco BRL, Grand Island, NY, USA). To prevent growth of glial cells 10 μM cytosine arabinoside (Sigma, St. Louis, MO, USA) (MW 300,000) was added to the cultures 40 h after seeding. The cells were left for 7 days in culture to differentiate and thereafter exposed to 25 μM hydrogen peroxide (Fluka, Buchs, Germany), 1 μM methylmercury (II) hydroxide (ALFA, Johnson Metthey, Karlsrhue, Germany) or 1 μM colchicine (Sigma, St.Louis, MO, USA) for 24 hrs. The caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (*O*-methyl) fluoromethyl ketone (z-VAD-fmk, Peptide Institute, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 100 mM, stored at –20°C and diluted 1/1 (v/v) in absolute ethanol before use. CGC were pre-incubated with 100 μM z-VAD-fmk 30 min before the treatment with colchicine, MeHg or hydrogen peroxide.

Nuclear Stainings

In order to evaluate nuclear morphology CGC, grown on coverslips, were fixed in ice-cold 80% methanol, washed in phosphate-buffered saline (PBS), stained with propidium iodide (Molecular Probes Eugene, Oregon, USA) (5 μg/ml) for 5 min and rinsed in PBS. Coverslips were mounted onto glass slides with PBS-glycerol (1:9) containing 0.1% phenylenediamine (Sigma, St. Louis, MO, USA) and examined using a fluorescence microscope. The percentage of nuclei with chromatin condensation was determined for each treatment by scoring no less than 300 nuclei in three fields for each culture dish. Statistical analysis was performed with One-Way Analysis of Variance (ANOVA), using the Tukey-Kramer multiple comparisons test (GraphPad Instat program). In addition, living cells were stained with propidium iodide and Hoechst 33342. Uptake of propidium iodide indicates membrane damage, a feature characteristic of necrosis, while Hoechst 33342 readily traverse the membrane of intact cells. Consequently, nuclear condensation visualised with Hoechst 33342, in the absence of propidium iodide uptake, is characteristic of apoptotic cells. However, in cell culture systems where phagocytosis of apoptotic cells does not occur, there is a delayed lysis of the plasma membrane of apoptotic cells (secondary necrosis), and some cells show condensed nuclei in addition to membrane permeability. Cells exposed to the stimuli inducing apoptosis were incubated with Hoechst 33342 and propidium iodide (= 0.5 μg/ml each in culture medium) for 10 min prior examination under a fluorescence microscope. A total of at least 200 cells were counted.

Staining with TUNEL (terminal deoxyribonucleotide transferase [TdT]-mediated dUTP nick end labeling)

Cells were grown on coverslips, treated with colchicine, MeHg or H_2O_2 for 24 h and fixed with methanol as described above. Cells were then covered with reaction mixture [20 μM dUTP-biotinylated, 0.25 U/μl terminal deoxyribonucleotide transferase, 1 mM $CoCl_2$, 25 mg/ml BSA (bovine serum albumin), 200 mM potassium cacodylate, 25 mM Tris-HCl pH 6.6 (Boehringer Mannheim, Bromma, Sweden)] and incubated at 37 °C for 1 h. Coverslips were then incubated in 300 mM NaCl, 30 mM sodium citrate at room temperature for 15 minutes. After washing twice with PBS, the samples were blocked with 2% BSA in PBS for 30 min at room temperature, then rinsed with PBS twice, covered with Extravidine-FITC (fluorescein isothiocyanate) (Sigma, St. Louis, MO) diluted 1/100 in PBS and incubated at 37°C for 30 min in the dark. After 2 washes with PBS, the coverslips were stained with propidium iodide (2.5 μg/ml in PBS) and mounted onto slides as described above for microscopic analysis. Images were collected with the C4742–95–10sc digital camera (Hamamatsu Photonics Norden AB, Solna, Sweden).

MTT Assay

The cytotoxic potential of colchicine, MeHg and H_2O_2 was evaluated with a colorimetric assay, based on the capacity of mitochondrial enzymes to transform the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a blue formazan product. This assay is useful for determining cell survival and function of mitochondrial enzymes (Slater, *et al.*, 1963; Mosmann, 1983; Ankarcrona *et al.*, 1995). CGC were treated with the toxic agents for 24 h, then the medium was removed and the cells were covered with MTT tetrazolium dissolved in serum free culture medium without phenol red to a final concentration of 0.3 mg/ml. After incubation at 37 °C for 1 h, the medium was removed and isopropanol was added to extract the formazan crystals. The absorbance of the samples was measured at $\lambda = 592$ nm on a Multiscan reader. Data (percentage of inhibition relative to untreated control) are means ± SEM of four independent determinations.

Measurement of Caspase Activity

Cleavage of the synthetic caspase substrate Ac-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (DEVD-MCA) was measured according to a method previously described (Nicholson *et al.*, 1995), with some modifications (Gorman *et al.*, 1999). At the end of the exposure to the various treatments the CGC were scraped gently from the plates, collected in tubes and washed with PBS. The pellets were suspended in PBS (100,000 cells/μl) and the cell suspensions were transferred in separate wells of a 96-well plate (25 μl/well). The plate was frozen on liquid nitrogen. Immediately before the assay, 50 μl of assay buffer {100 mM N-2-hydroxyethyl-piperazine-N′-2-ethanesulphonic acid (HEPES) pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate, 5 mM dithiothreitol (DTT), 10^{-4}% Nonidet P-40, 50 μM DEVD-MCA} was added to each well. The caspase substrate DEVD-MCA was purchased from Peptide Institute (Osaka, Japan), dissolved in DMSO at the concentration of 200 mM and stored at –20 °C. Substrate cleavage leading to the release of free MCA was monitored at 37 °C using a Fluoroskan II (Labsystem AB, Stockholm, Sweden) (excitation 355 nm, emission 460 nm). Fluorescent units were converted to pmoles of MCA released using a standard curve generated with MCA and subsequently related to number of cells. The measurements were performed in triplicates and the experiments were repeated 3 times. Statistical analysis was performed with ANOVA, using the Dunnet's multiple comparisons test.

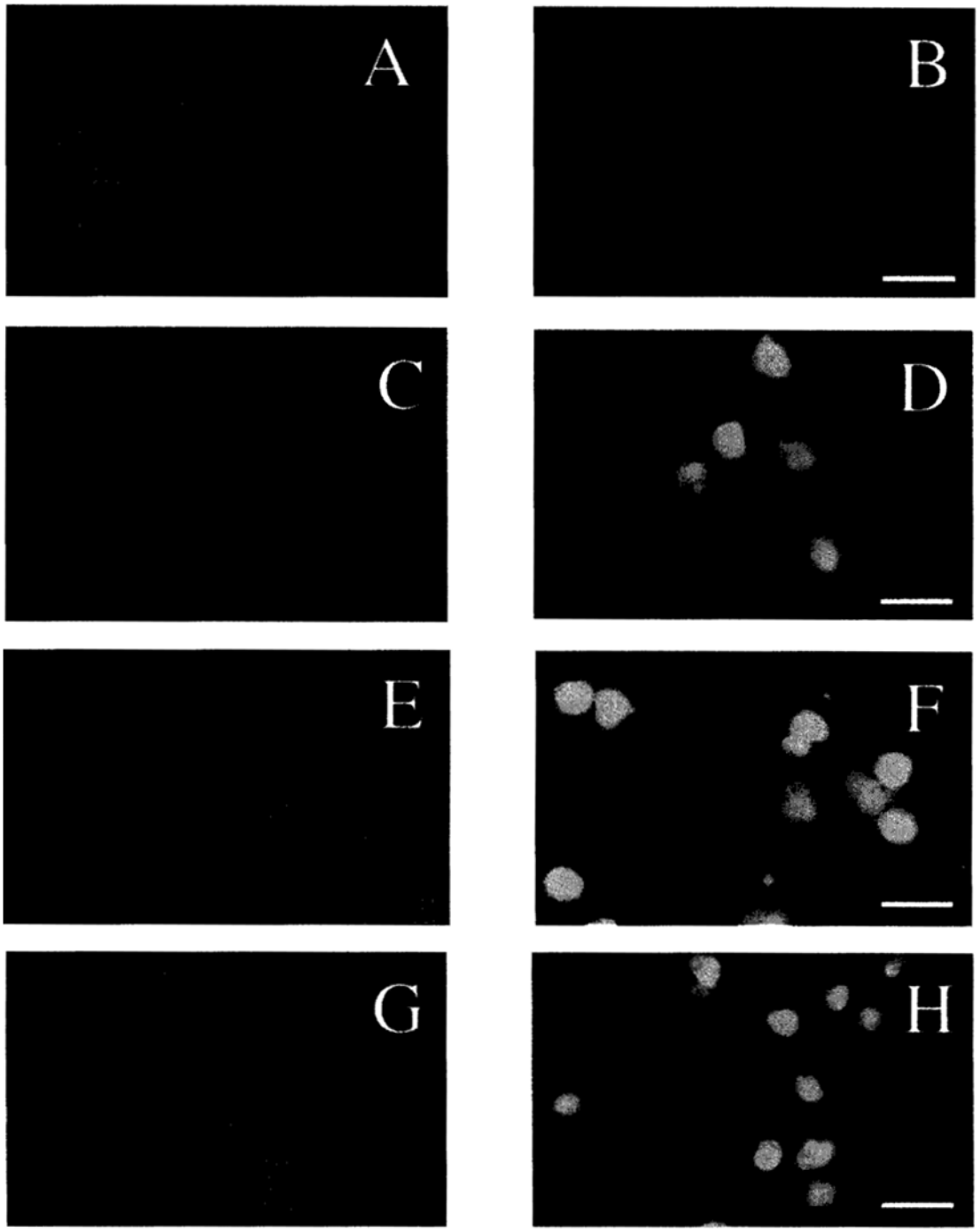

FIGURE 1 Double staining of CGC with propidium iodide (left) and TUNEL assay (right). Panel A and B, control cells. Nuclei with chromatin condensation and TUNEL positive cells were detected after exposure to 1 μM colchicine (C, D), 1 μM MeHg (E, F) and 25 μM hydrogen peroxide (G, H) for 24 h. Scale bar = 5 μm (See Color Plate V at the back of this issue)

Protein Extraction, SDS-PAGE and Immunoblotting

CGC were scraped, centrifuged and washed with PBS. The cells were suspended in gel loading buffer (0.4% SDS, 4% glycerol, 1% β-mercaptoethanol, 12.5 mM Tris-HCl, pH 6.8) and boiled for 5 min. The proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (Laemmli, 1970) in Tris-glycine buffer, pH 8.3. Proteins were quantified by a procedure described previously by Schaffner-Weissmann (Schaffner and Weissmann, 1973) and equivalent amounts of proteins (40 μg) were loaded in the wells. The proteins were then electroblotted onto nitrocellulose membranes at 75 V for 1–2 h using as transfer buffer 20% (v/v) methanol, 186 mM glycine, 26 mM Tris, pH 8.3. In order to verify equal loading, the proteins transferred on the membranes were visualized with 2% red Ponceau-S, 3% trichloroacetic acid. The blots were blocked with a solution containing 5% non-fat dry milk, 1% BSA in HSB (500 mM NaCl, 50 mM Tris-HCl, pH 7.5), at 4 °C overnight. The membranes were then incubated with a polyclonal antibody against the p17 fragment of caspase-3 (a kind gift from Dr. D. Nicholson), diluted 1/5000 in a solution containing 1% BSA in HSBT (0.05 % Tween-20 in HSB) at room temperature for 1 h. After washing 3 times for 15 minutes with HSBT, the blots were incubated with a goat anti-rabbit IgG-peroxidase conjugated (Pierce, Rockford, Illinois, USA), diluted 1/10,000 in 1% BSA in HSBT, at room temperature for 1 h. They were then washed again 3 times as described above, rinsed twice for 5 min with 150 mM NaCl, 50 mM Tris, pH 7.5, incubated with ECL reagents for chemiluminescence (Amersham, Little Chalfont, Bucks, UK) and exposed to X-ray autoradiography films (Fuji, Japan).

Immunocytochemistry

CGC grown on coverslips were fixed with 4% ice-cold paraformaldehyde for 20 minutes. The coverslips were incubated with a polyclonal antibody against the active fragment of caspase-3, p17 (Kouroku *et al.*, 1998; Urase *et al.*, 1998) diluted 1/200 in PBS supplemented with 0.3% Triton-X100 and 0.5% BSA, in a humid chamber at 4°C overnight. The cells were then rinsed with PBS, and incubated with a FITC-conjugated secondary antibody (Amersham) diluted 1/10 in PBS containing 0.3% Triton-X100 for 30 min at room temperature. After 3 rinses with PBS, coverslips were mounted in glycerol-PBS containing 0.1% phenylenediamine. The CGC were then examined with a fluorescence microscope Olympus BX60. Images were collected with the C4742–95–10sc digital camera.

RESULTS

The cascades of cellular events leading to cell death of cerebellar granule neurons exposed to colchicine, MeHg or hydrogen peroxide were evaluated and compared. The results are presented in a way to highlight the similarities and the differences in morphological and biochemical changes occurring in cells exposed to the selected stimuli. The data are summarised in table II.

Analysis of Morphology

Cell shrinkage and loss of neurites were observed in CGC exposed to 1 μM colchicine (Bonfoco et al., 1995; Gorman et al., 1999), 1 μM MeHg (Daré et al., 2000) or 25 μM hydrogen peroxide (Götz et al., 1999; Ahlbom et al., 2000) for 24 h. Staining of the nuclei with propidium iodide revealed the presence of condensed chromatin in a large proportion of the treated cells (Fig. 1, panel C, E, G). Chromatin changes were accompanied by convolution of the nuclear outline. These observations indicated that all of the three toxic stimuli induced morphological changes typical of cells undergoing apoptosis.

Furthermore, staining of living cells with Hoechst 33342 and propidium iodide revealed that a number of cells (less than to 15%) exposed to colchicine, MeHg or hydrogen peroxide exhibited membrane permeability to propidium iodide in association with nuclear condensation, indicating that they had undergone secondary necrosis (data not shown).

TUNEL Assay

Exposure to colchicine, MeHg and hydrogen peroxide for 24 h had been previously shown to induce DNA cleavage into high molecular weight fragments of 700, 300 and 50 kbp, using field inversion gel electrophoresis (FIGE) (see Gorman *et al.*, 1999; Ahlbom *et al.*, 1999; Götz *et al.*, 1999; Daré *et al.*, 2000). The presence of DNA fragments with 3'-hydroxyl ends due to activation of specific endonucleases is often observed in cells undergoing apoptosis, and can be evaluated in fixed cells using the TUNEL assay. Figure 1 shows that TUNEL positive cells were induced by exposure to 1 μM colchicine (panel D), 1 μM MeHg (panel F) or 25 μM hydrogen peroxide (panel H) for 24 h. However, not all cells showing chromatin condensation were positive for TUNEL, whereas all TUNEL positive cells presented condensed chromatin.

MTT Assay

Mitochondrial metabolism of MTT into formazan was measured in CGC exposed to the toxic agents for 24 h. Decreased MTT metabolism was observed after exposure to 1 μM colchicine (31.2 ± 9.4 % inhibition), 1 μM MeHg (37.6 ± 6.6 % inhibition) and 25 μM hydrogen peroxide (37.9 25 ± 7.8 % inhibition), as compared to control cells.

DEVDase Activity Assay

The main effector caspases (caspase-3 and 7) of apoptosis have a preference for cleaving after the sequence DEVD and therefore, the ability of the various treatments to stimulate DEVDase activity was examined. Colchicine was previously shown to cause a rapid (within 6 h) induction of DEVDase activity, which continued to increase in the following hours (Gorman *et al.*, 1999). After 18 h of treatment DEVDase activity was 18-fold that of basal. This demonstrated a clear involvement of caspases in colchicine-induced CGC death. In contrast, the level of DEVDase activity measured in cells treated with 1 μM MeHg was found not to be significantly different from control cells (Daré *et al.*, 2000).

In Fig. 2 we present a time course of DEVDase activity following exposure to 25 μM hydrogen peroxide. There was no increase in DEVDase activity detected at any of the time points tested in cultures treated with hydrogen peroxide (Fig. 2, A), in spite of the progressive chromatin condensation (Fig. 2, B). In fact, there was a slight but significant ($p < 0.05$ at 0.5 h and $p < 0.01$ at 6 h) decrease in the basal level of activity, suggesting that hydrogen peroxide may inhibit the DEVDase activity.

TABLE I The caspase inhibitor z-VAD-fmk protects CGC against colchicine, but not against MeHg or hydrogen peroxide

Treatment	*– z-VAD-fmk*[a]	*+ z-VAD-fmk*[ab]
Control	3.3 ± 0.9	6.7 ± 1.4
1 μM colchicine	60.3 ± 4.6[c]	16.3 ± 7.8[d]
1 μM MeHg	57.3 ± 7.2[c]	59.7 ± 2.0[c]
25 μM hydrogen peroxide	51.3 ± 2.7[c]	63.3 ± 2.0[c]

a. Values (means ± SEM, n = 3) show the percentage chromatin condensation in the CGC cultures following 24 h exposure to the various treatments. Statistical analysis was performed with ANOVA [$F(7, 16) = 45.9$; $p < 0.0001$] followed by the Tukey-Kramer multiple comparisons test
b. z-VAD-fmk (100 μM) was added to the conditioned medium 30 min before exposure to colchicine, MeHg or hydrogen peroxide.
c. Significantly different from control ($p < 0.001$).
d. Significantly different from colchicine in absence of z-VAD-fmk ($p < 0.001$).

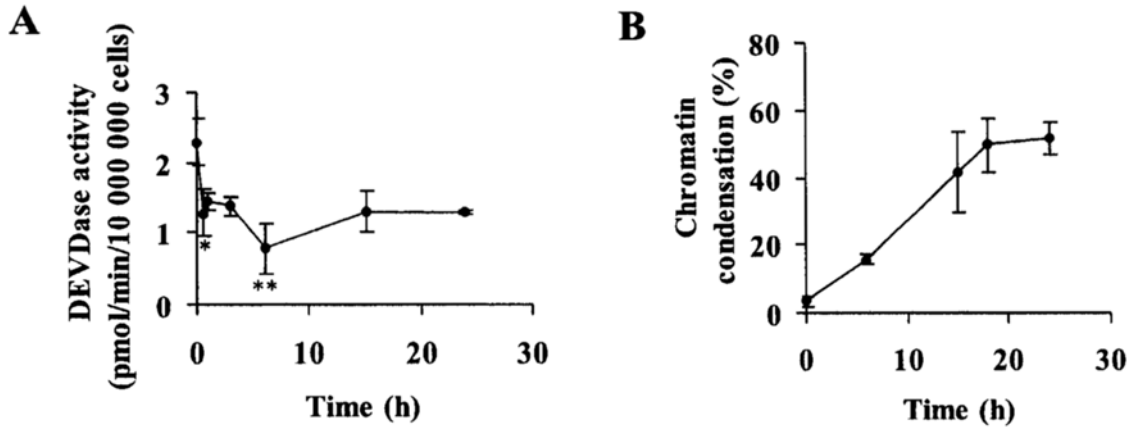

FIGURE 2 Lack of caspase activity in CGC treated with hydrogen peroxide. (A) DEVD-MCA cleavage activity was measured in cellular extracts taken at different times following exposure to 25 μM hydrogen peroxide using a fluorimetric assay. Values are means ± SEM of three determinations. Statistical analysis was performed with ANOVA ($F = 3.349$, $p = 0.0292$), with Dunnet's post-hoc test (*significantly different from control, $p \leq 0.05$; **significantly different from control, $p \leq 0.01$). (B) Time course of nuclear condensation. Nuclei stained with propidium iodide were scored for nuclear condensation in cultures treated with 25 μM hydrogen peroxide for 24 h. Values are means ± SEM of three independent determinations

Detection of Activated Caspase-3 by Western blotting and Immunocytochemistry

Caspase-3 is one of the main group II caspases responsible for DEVD cleavage activity (i.e., effector caspase activity) in apoptotic cells. Activation of procaspase-3 occurs by proteolytic cleavage and generates the active p17 fragment. Analysis of protein extracts obtained from CGC exposed to 1 μM colchicine by Western blotting confirmed that the level of p17 increased progressively starting from 6 h (Gorman *et al.*, 1999) (Fig. 3). The p17 fragment was also detectable in CGC exposed to 1 μM MeHg for 24 h (Daré *et al.*, 2000). However, p17 was not detectable in CGC exposed to 25 μM hydrogen peroxide for 3–24 h (Fig. 3).

Measurement of DEVDase activity in whole cell extracts or detecting p17 by immunoblotting are relatively crude ways to assess the activation of caspase-3, since it is possible that caspases may be activated in only a small proportion of the total cell population at any one time and then rapidly inactivated. Therefore, it was decided to examine the presence of activated caspase-3 in single cells by performing immunocytochemistry. Using an antibody that recognizes only p17 and not its pro-form, activated caspase-3 was found to be abundant in the majority of CGC exposed to colchicine (Fig. 4, A), while it was virtually undetectable in control cells (results not shown). CGC exposed to MeHg showed sporadic staining with the p17 antibody (Fig. 4, B). In contrast, the activated p17 fragment was undetectable in CGC exposed to hydrogen peroxide (Fig. 4, C).

Effect of Caspase Inhibitors

The pan-caspase inhibitor z-VAD-fmk inhibits both group II and group III caspases. Previous experiments have demonstrated that z-VAD-fmk strongly protected CGC from colchicine-induced chromatin condensation (Gorman *et al.*, 1999), but not from MeHg-induced chromatin condensation (Daré *et al.*, 2000). Here we report that the presence of z-VAD-fmk had no protective effect also against hydrogen peroxide-induced chromatin condensation (Table I).

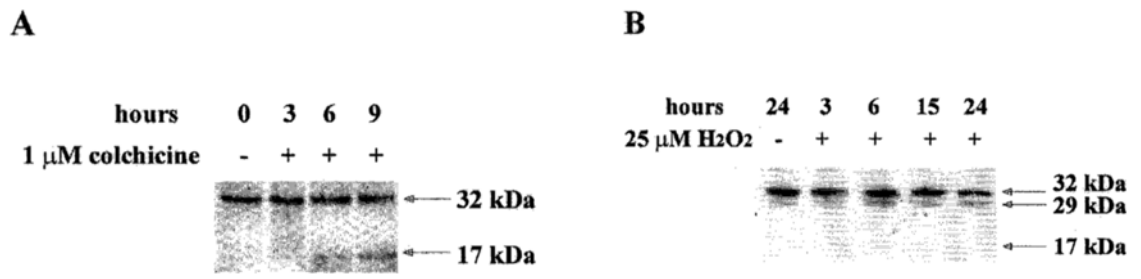

FIGURE 3 Detection of the activated p17 fragment of caspase-3 by Western blotting. Total proteins extracted from CGC at different time points following exposure to either 1 μM colchicine (A) or 25 μM hydrogen peroxide (B) were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The p17 antibody used in this experiment recognizes pro-caspase 3 (32 kDa), the activated p17 fragment and an additional p29 fragment. The p17 fragment was detectable in cells exposed to colchicine for 6–9 h (A) but absent in cells exposed to hydrogen peroxide for 3–24 h (B)

DISCUSSION

In the studies presented in this paper, we have exposed CGC to three different conditions, i.e., colchicine, hydrogen peroxide and MeHg. All of the treatments induced apoptosis according to the classical morphological criteria based on shrinkage of the cells, chromatin condensation and DNA cleavage into high molecular weight fragments of ~50 kbp (Gorman *et al.*, 1999; Ahlbom *et al.*, 1999; Götz *et al.*, 1999; Daré *et al.*, 2000). Chromatin condensation, associated with the initial step of DNA cleavage forming HMW-DNA fragments, is considered an early event of apoptosis, which occurs independently from the subsequent and dispensable internucleosomal DNA fragmentation (Hara *et al.*, 1996). Therefore, DNA stainings (i.e. propidium iodide) and techniques to detect DNA fragmentation, such as TUNEL, do not provide the same type of information. This may explain the observation made by us and others (Kaasik *et al.*, 1999) that not all cells with condensed chromatin are positive for TUNEL.

The data summarized in Table II clearly indicate that despite the common nuclear changes induced by these neurotoxic agents there was a range of responses in terms of caspase involvement in the process of cell death. It is clear that only colchicine induced distinct caspase activation. The caspase inhibitor z-VAD-fmk significantly reduced chromatin condensation induced by colchicine indicating a causal role of caspases in the process of cell death. However, it did not prevent the damage to the neuronal processes, as also reported by Volbracht *et al.* (1999).

The cell death pathways promoted by the other two agents are less certain. There is a distinct lack of evidence for an involvement of caspases in CGC death due to hydrogen peroxide, since the caspase-3 like activity was lower than in control cells and the caspase inhibitor z-VAD-fmk did not block chromatin condensation. There is a critical redox active cysteine in caspases that is easily inhibited by oxidative stress (Baker *et al.*, 2000). In fact, hydrogen peroxide has previously been shown to inhibit activated caspases in Jurkat T lymphocytes (Hampton and Orrenius, 1997) although paradoxically, depending on the concentration and cell type it can also lead to caspase-mediated apoptosis (Stridh *et al.*, 1998). Evidence has also been presented suggesting that hydrogen peroxide might inhibit apoptosis by depletion of ATP (adenosine-5'-triphosphate) (Lee and Shacter, 1999). Caspases did not appear significantly activated during MeHg-induced CGC death, and the caspase inhibitor z-VAD-fmk could not prevent chromatin condensation. MeHg is known to increase generation of reactive oxygen species (Sarafian and Verity, 1991) and this may account for the low levels of active caspases observed.

TABLE II Summary of parameters affected by exposure to colchicine, MeHg or hydrogen peroxide in CGC

Treatment[a]	*Apoptotic nuclei*[b]	*H.M.W.-DNA fragments*[c]	*TUNEL positive cells*	*MTT metabolism*	*DEVDase activity*	*p17 detection immunoblotting*	*p17 detection ICC*	*Protection with z-VAD-fmk*[d]	*Protection with antioxidants*[e]
1 μM colchicine[f]	61.8 ± 8.9	increase	present	decrease	increase	+ + +	+ + +	yes	no
1 μM MeHg[g]	67.1 ± 8.1	increase	present	decrease	no increase	+	+	no	yes
25 μM H_2O_2[h]	56.5 ± 6.4	increase	present	decrease	no increase	–	–	no	yes

a. Values were obtained in different experiments where CGC at DIV 7 were exposed for 24 h in conditioned medium.
b. Fixed cells were stained with PI and nuclei with chromatin condensation were scored. Values (mean ± SEM) from 3–4 individual experiments are shown.
c. DNA fragmentation was evalulated by FIGE. Approximate fragment sizes: 700 kbp, 300 kbp and 50 kpb.
d. Protection was evaluated by scoring cells with chromatin condensation after 24 h exposure to the toxic agent in the precence or absence of the pan-caspase inhibitor z-VAD-fmk (100 μM).
e. Protection was evaluated after 24 h exposure to the toxic agent in the precence or absence of the radical scavenger J811 (10 μM), using morphological analysis of the cells at the phase contrast miscroscope, scoring PI-stained cells with chromatin condensation and determining DNA fragmentation with FIGE.
f. From Bonfoco *et al.*, 1995, Ahlbom *et al.*, 1999, Gorman *et al.*, 1999, Götz *et al.*, 1999, Ahlbom *et al.*, 2000 and present paper (TUNEL assay and immunocytochemistry).
g. From Daré *et al.*, 2000, and present paper (TUNEL assay, MTT assay and immunocytochemistry).
h. From Götz *et al.*, 1999, Ahlbom *et al.*, 2000 and from the present paper (TUNEL assay, MTT assay, DEVDase activity, p17 detection and z-VAD-fmk protection).

In contrast to the lack of protection by z-VAD-fmk, antioxidants such as estrogens, protected CGC exposed to either MeHg and hydrogen peroxide. Estrogens have been shown to display antioxidant properties, based on their phenolic A-ring, which confers part of the radical scavenging capacity (Mooradian, 1993; Behl *et al.*, 1997; Moosmann and Behl, 1999). Pre-incubation of CGC with either 17β-estradiol or the radical scavenger J811 (Römer *et al.*, 1997), a synthetic compound structurally derived from estradiol, prevented cell shrinkage, loss of neurites, chromatin condensation and DNA fragmentation induced by MeHg (Table II) (Daré *et al.*, 2000). J811 also blocked cell shrinkage, loss of neurite arborization, chromatin condensation and DNA cleavage in CGC exposed to hydrogen peroxide, while it was completely ineffective against colchicine (Gotz et *al.*, 1999). This points to reactive oxygen species formation as the crucial step in the process of cell death due to MeHg and hydrogen peroxide, whilst it indicates that caspases are not relevant to the execution of death following oxidative stress in CGC.

It is probable that hydrogen peroxide and MeHg-induced CGC death may involve proteases other than caspases. In support of this we have found that MeHg and, to a minor extent, hydrogen peroxide, consistently increased proteolytic fragments obtained by specific calpain cleavage of procaspase-3 (29 kDa) (Wolf *et al.*, 1999; McGinnis *et al.*, 1999) and α-fodrin (150 kDa) (Nath *et al.*, 1996), pointing to activation of calpain (Daré *et al.*, 2000). Calpains are proteases regulated by Ca^{2+} (Sorimachi *et al.*, 1997), thus the increase of intracellular Ca^{2+} that is well documented in MeHg-exposed CGC might be responsible for calpain activation (Marty and Atchison, 1997). It has recently been reported that calpain activity can cause inactivation of caspase-3 protease activity in hippocamal neurons (Lankiewicz *et al.*, 2000) although the neurons can still die. A similar effect may be occurring in CGC treated with either hydrogen peroxide or MeHg which would explain the lack

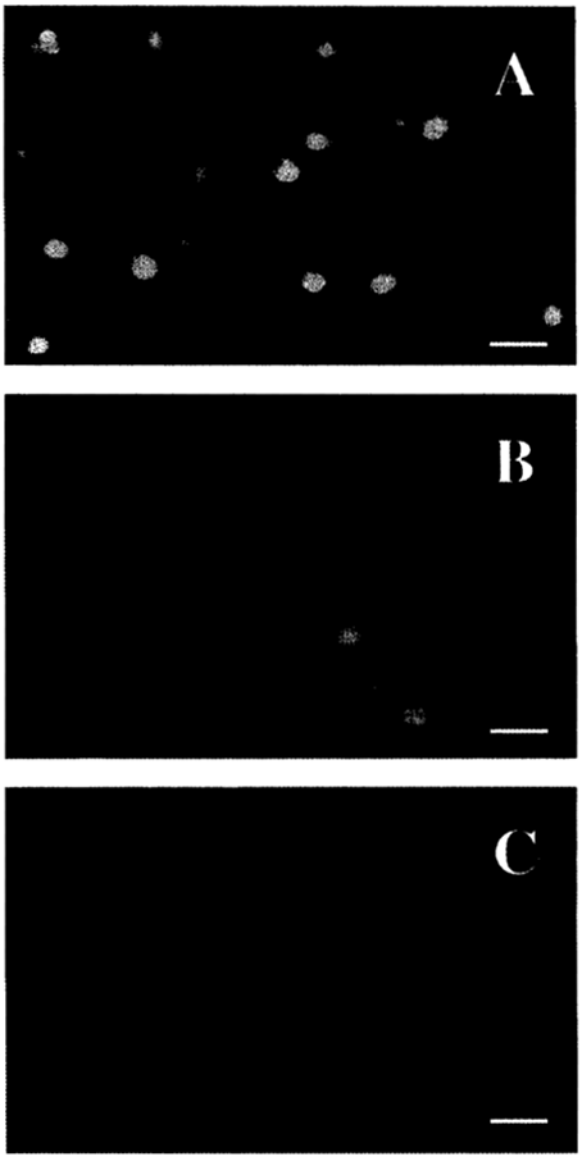

FIGURE 4 Immunocytochemical staining with the p17 antibody, specific for activated caspase-3. CGC exposed to colchicine (A), MeHg (B) or hydrogen peroxide (C). Scale bar = 5 μm (See Color Plate VI at the back of this issue)

or low levels of DEVDase activity and lack or low levels of p17 fragment for each of the treatments respectively. The fact that the scavestrogen J811 prevented the calpain dependent formation of α-fodrin and procaspase-3 break-

down products induced by MeHg points to calpain activation being a consequence of oxidative stress (Daré *et al.*, 2000).

Other groups have also described the induction of morphological changes typical of apoptosis in the absence of caspase activation. For example, retinal cells have been reported to undergo cell shrinkage, DNA nicking (TUNEL assay) and phosphatidylserine exposure in the absence of caspase activity while inhibitors of caspase activity had no effect on cell survival (Carmody and Cotter, 2000). The anticancer agent, curcumin, has also been shown to cause cell death characterized by chromatin condensation and high molecular weight DNA fragmentation with a lack of involvement of caspases (Piwocka *et al.*, 1999). These findings prompt the question as to how chromatin condensation and DNA fragmentation are achieved in CGC without the action of caspases. It has recently been shown that a number of different factors contribute to the nuclear changes that are observed during apoptosis. Among them caspase-activated DNase (CAD) and apoptotic chromatin condensation inducer in the nucleus (Acinus) cause chromatin condensation in a caspase-3 dependent manner, whereas apoptosis-inducing factor (AIF) does not require caspase activity. AIF has been characterized as a 57 kDa flavoprotein that is normally confined to the mitochondria but translocates to the nucleus when apoptosis is induced (Susin *et al.*, 1999). AIF is particularly interesting to the present study in that it is capable of inducing both chromatin condensation and high molecular weight DNA fragmentation in a caspase-independent manner. It could therefore be responsible for the nuclear changes observed in CGC treated with MeHg and H_2O_2.

A possible involvement of lysosomes in oxidative stress-induced cell death has lately attracted considerable attention. Several studies have demonstrated that intracellular generation of reactive oxygen species causes early damage of lysosomal membranes and results in spillage of lysosomal hydrolytic enzymes in the cytosol (Ollinger and Brunk, 1995; Roberg *et al.*, 1999). The intensity of the toxic insult reaching the lysosomes seems to influence the cell fate, leading alternatively to repairable sub-lethal damage or cell death (Brunk *et al.*, 1997). The endonucleases and proteases released into the cytosol from the lysosomes may be execution factors in the processes of chromatin condensation and DNA fragmentation occurring in cells exposed to oxidative stress.

In conclusion, the data summarized here strongly suggest that there is more than one pathway operating in CGC leading to apoptotic cell death. Although caspase activation is frequently considered to be a biochemical hallmark of apoptosis, the evidence presented indicates that morphological changes and DNA fragmentation characteristic of apoptosis may not be always associated with caspase activation. An understanding of the pathways activated by neurotoxicants appears indispensable to development of appropriate therapeutic strategies for neuroprotection.

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References

Ahlbom, E., Grandison, L., Bonfoco, E., Zhivotovsky, B. and Ceccatelli, S. (1999) Androgen treatment of neonatal rats decreases susceptibility of cerebellar granule neurons to oxidative stress in vitro. *Eur. J. Neurosci*, **11**, 1285–1291.

Ahlbom, E., Gogvadze, V., Chen M., Celsi, G. and Ceccatelli, S. (2000) Prenatal exposure to high levels of glucocorticoids increases the susceptibility of cerebellar granule cells to oxidative stress-induced cell death. *Proc. Natl. Acad. Sci. (USA)*, **97**, 14726–14730.

Ankarcrona, M., Dypbukt, J.M., Bonfoco, M., Zhivotovsky, B., Orrenius, S., Lipton, S.A. and Nicotera, P. (1995)

Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron*, **15**, 961–973.

Baker, A., Santos, B.D., Powis, G. (2000) Redox control of caspase-3 activity by thioredoxin and other reduced proteins. *Biochem. Biophys. Res. Commun.* **268**, 78–81.

Behl, C., Skutella, T., Lezoualc'h, F., Post, A., Widmann, M., Newton, C.J. and Holsboer, F. (1997) Neuroprotection against oxidative stress by estrogens: structure-activity relationship. *Mol. Pharmacol.* **51**, 535–541.

Behl, C. (1999) Alzheimer's disease and oxidative stress: implications for novel therapeutic approaches. *Prog. Neurobiol.* **57**, 301–323.

Bonfoco, E., Ceccatelli, S., Manzo, L. and Nicotera, P. (1995) Colchicine induces apoptosis in cerebellar granule cells. *Exp. Cell Res.* **218**, 189–220.

Brunk, U. T.; Dalen, H.; Roberg, K.; Hellquist, H. B. (1997) Photo-oxidative disruption of lysosomal membranes causes apoptosis of cultured human fibroblasts. *Free Radic. Biol. Med.* **23**, 616–626.

Carmody, R.J. and Cotter, T.G. (2000) Oxidative stress induces caspase-independent retinal apoptosis *in vitro*. *Cell Death Diff.* **7**, 282–291.

Castoldi, A.F., Barni, S., Turin, I, Gandini, C. and Manzo, L. (2000) Early acute necrosis, delayed apoptosis and cytoskeletal breakdown in cultured cerebellar granule neurons exposed to methylmercury. *J. Neurosci. Res.* **59**, 775–787.

Cookson, M.R. and Shaw, P.J. (1999) Oxidative stress and motor neuron disease. *Brain Pathol.* **9**, 165–186.

Daré, E., Götz, M.E., Zhivotovsky, B., Manzo, L. and Ceccatelli, S. (2000) The antioxidants J811 and 17β-estradiol protect cerebellar granule cells from methylmercury-induced apoptotic cell death. *J. Neurosci. Res.* **62**, 557–565.

Gorman, A.M., McGowan, A. and Cotter, T.G. (1996) Oxidative stress and apoptosis in neurodegeneration. *J. Neurol. Sci.* **139**, S45–52.

Gorman, A.M., Orrenius, S. and Ceccatelli, S. (1998) Apoptosis in neuronal cells: role of caspases. *Neuroreport* **9**, R49-R55.

Gorman, A.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S. and Ceccatelli, S. (1999) Cytochrome *c* release and caspase-3 activation during colchicine-induced apoptosis of cerebellar granule cells. *Eur. J. Neurosci.* **11**, 1067–1073.

Götz, M.E., Ahlbom, E., Zhivotovsky, B., Blum-Degen, D., Oettel, M., Römer, W., Riederer, P., Orrenius S. and Ceccatelli, S. (1999) Radical scavenging compound J 811 inhibits hydrogen peroxide-induced death of cerebellar granule cells. *J. Neurosci. Res*, **56**, 420–426.

Hampton, M.B. and Orrenius S. (1997) Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. *FEBS Lett.* **414**, 552–556.

Hara, S., Halicka, H., Bruno, S., Gong, J., Traganos, F. and Darzynkiewicz, D. (1996) Effect of protease inhibitors on early events of apoptosis. *Exp. Cell. Res.* **223**, 372–384.

Jenner, P. and Olanow, C.W. (1996) Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology* **47**, S161–170.

Kaasik, A., Vassiljev, V., Poldoja, E., Kalda, A. and Zharkovsky, A. (1999) Do nuclear condensation or fragmentation and DNA fragmentation reflect the mode of neuronal death? *NeuroReport* **10**, 1937–1942.

Kerr, J.F.R., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 1790–1794.

Kouroku, Y., Urase, K., Fujita, E., Isahara, K., Ohsawa, Y., Uchiyama, Y., Momoi, M.Y. and Momoi, T. (1998) Detection of activated caspase-3 by a cleavage site-directed antiserum during naturally occurring DRG neurons apoptosis. *Biochem. Biophys Res. Commun.* **247**, 780–784.

Kunimoto, M. (1994) Methylmercury induces apoptosis of rat cerebellar neurons in primary culture. *Biochem. Biophys. Res. Commun.* **204**, 310–317.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685,.

Lankiewicz, S., Marc Luetjens, C., Truc Bui, N., Krohn, A.J., Poppe, M., Cole, G.M., Saido, T.C. and Prehn, J.H. (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. *J. Biol. Chem.* **275**, 17064–17071.

Lee, Y. and Shacter, E. (1999) Oxidative stress inhibits apoptosis in human lymphoma cells. *J. Biol. Chem.* **274**, 19792–19798.

Marty, M.S. and Atchison, W.D. (1997) Pathways mediating Ca^{2+} entry in rat cerebellar granule cells following in vitro exposure to methyl mercury. *Toxicol. Appl. Pharmacol.* **147**, 319–330.

Mattson, M.P. (1992) Effects of microtubule stabilization and destabilization on tau immunoreactivity in cultured hippocampal neurons. *Brain Res.* **582**, 107–118.

McGinnis, K.M., Gnegy, M.E., Park, Y.H., Mukerjee, N. and Wang, K.K. (1999) Procaspase-3 and poly(ADP)ribose polymerase (PARP) are calpain substrates. *Biochem. Biophys. Res. Commun.* **263**, 94–99.

Mooradian, A.D. (1993) Antioxidant properties of steroids. *J. Steroid Biochem. Mol. Biol.* **45**, 509–511.

Moosmann, B. and Behl, C. (1999) The antioxidant neuroprotective effects of estrogens and phenolic compounds are independent from their estrogenic properties. *Proc. Natl. Acad. Sci. USA* **96**, 8867–8872.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* **65**, 55–63.

Nakagawa, Y., Nakamura, S., Kasé, Y., Noguchi, T. and Ishihara, T. (1987) Colchicine lesions in the rat hippocampus mimic the alterations of several markers in Alzheimer's disease. *Brain Res.* **408**, 57–64.

Nath, R., Raser, K.J., Stafford, D., Hajimohammadreza, I., Pasner, A., Allen, H., Talanian, R.V., Yuen, P., Gilbertsen, R.B. and Wang, K.K.W. (1996) Non-erythroid α-spectrin breakdown by calpain and interleukin 1β-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis. *Biochem. J.* **319**, 683–690.

Nicholson D.W., Ali A., Thornberry N.A., Vaillancourt J.P., Ding C.K., Gallant M., Gareau Y., Griffin P.R., Labelle M., Lazebnik Y.A., Munday N.A., Raju A.M., Smulson M.E., Yamin T.T., Yu V.L. and Miller D.K. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37–43.

Nicholson, D.W. and Thornberry, N.A. (1997) Caspases: killer proteases. *Trends Biochem. Sci.* **22**, 299–306.

Ollinger, K. and Brunk, U. T. (1995) Cellular injury induced by oxidative stress is mediated through lysosomal damage. *Free Radic. Biol. Med.* **19**, 565–574.

Piwocka, K., Zablocki, K., Wieckowski, M.R., Skierski, J., Feiga, I., Szopa, J., Drela, N., Wojtczak, L. and Sikora, E. (1999) A novel apoptosis-like pathway, independent of mitochondria and caspases, induced by curcumin in human lymphoblastoid T (Jurkat) cells. *Exp. Cell Res.* **249**, 299–307.

Roberg, K., Johansson, U. and Ollinger, K. (1999) Lysosomal release of cathepsin D precedes relocation of cytochrome c and loss of mitochondrial transmembrane potential during apoptosis induced by oxidative stress. *Free Radic. Biol. Med.* **27**, 1228–1237.

Römer, W., Oettel, M., Droescher, P. and Schwarz, S. (1997) Novel "scavestrogens" and their radical scavenging effects, iron-chelating, and total antioxidative activities: Δ8,9-dehydro derivatives of 17 α-estradiol. *Steroids* **62**, 304–310.

Sarafian, T. and Verity, M.A. (1991) Oxidative mechanisms underlying methyl mercury neurotoxicity. *Int. J. Dev. Neurosci.* **9**, 147–153.

Schaffner, W. and Weissmann, C. (1973) A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **56**, 502–514.

Schousboe, A., Meier, E., Drejer, J. and Hertz, L. (1989) Preparation of cultures of mouse (rat) cerebellar granule cells. In: Shahar, A., de Vellis, J., Vernadakis, A. and Haber, B (Ed), *Dissection and Tissue Culture Manual of the Nervous System* (A.R. Liss, New York), pp. 203–206.

Slater, T.F., Sawyer, B. and Strŝuli, U. (1963) Studies on succinate-tetrazolium reductase systems III. Points of coupling of four different tetrazolium salts. *Biochim. Biophys. Acta* **77**, 383–393.

Sorimachi, H., Ishiura, S. and Suzuki, K. (1997) Structure and physiological function of calpains. *Biochem. J.* **328**, 721–732.

Stridh, H., Kimland, M., Jones, D.P., Orrenius, S. and Hampton, M.B. (1998) Cytochrome *c* release and caspase activation in hydrogen peroxide- and tributyltin-induced apoptosis. *FEBS Lett.* **429**, 351–355.

Stroh, C. and Schulze-Osthoff, K. (1998) Death by a thousand cuts: an ever increasing list of caspase substrates. *Cell Death Differ.* **5**, 997–1000.

Sun, A.Y. and Chen, Y.M. (1998) Oxidative stress and neurodegenerative disorders. *J. Biomed. Sci.* **5**, 401–414.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Constantini, P, Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D., Penninger, J.M. and Kroemer, G. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441–446.

Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: enemies within. *Science* **281**, 1312–1316.

Urase, K., Fujita, E., Miho, Y. Kouroku, Y., Mukasa, T. Yagi, Y., Momoi, M.Y. and Momoi, T. (1998) Detection of activated Caspase-3 (CPP32) in the vertebrate nervous system during development by a cleavage site-directed antiserum. *Dev. Brain Res.* **111**, 77–87.

Volbracht, C., Leist, M. and Nicotera P. (1999) ATP controls neuronal apoptosis triggered by microtubule breakdown or potassium deprivation. *Mol. Med.* **5**, 477–89.

Wolf, B.B., Goldstein, J.C., Stennicke, H.R., Beere, H., Amarante-Mendes, G.P., Salvesen, G.S. and Green, D.R. (1999) Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. *Blood* **94**, 1683–1692.

Wyllie, A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284**, 555–556.