Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 70 (2005) 470–480
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Evaluation of the neuronal apoptotic pathways involved in cytoskeletal disruption-induced apoptosis

Elvira G. Jordà [a,1], Ester Verdaguer [b,1], Andrés Jimenez [a], S. Garcia de Arriba [b], Clemens Allgaier [b], Mercè Pallàs [a,2], Antoni Camins [a,*,2]

[a] *Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia, Universitat de Barcelona, Nucli Universitari de Pedralbes, E-08028 Barcelona, Spain*

[b] *Rudolf-Boehm-Institute of Pharmacology and Toxicology, University of Leipzig, Härtelstraße 16-18, D-04107 Leipzig, Germany*

Received 18 March 2005; accepted 29 April 2005

Abstract

The cytoskeleton is critical to neuronal functioning and survival. Cytoskeletal alterations are involved in several neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. We studied the possible pathways involved in colchicine-induced apoptosis in cerebellar granule neurons (CGNs). Although colchicine evoked an increase in caspase-3, caspase-6 and caspase-9 activation, selective caspase inhibitors did not attenuate apoptosis. Inhibitors of other cysteine proteases such as PD150606 (a calpain-specific inhibitor), Z-Phe-Ala fluoromethyl ketone (a cathepsins-inhibitors) and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (serine-proteases inhibitor) also had no effect on cell death/apoptosis induced by colchicine. However, BAPTA-AM 10 μM (intracellular calcium chelator) prevented apoptosis mediated by cytoskeletal alteration. These data indicate that calcium modulates colchicine-induced apoptosis in CGNs. PARP-1 inhibitors did not prevent apoptosis mediated by colchicine. Finally, colchicine-induced apoptosis in CGNs was attenuated by kenpaullone, a cdk5 inhibitor. Kenpaullone and indirubin also prevented cdk5/p25 activation mediated by colchicine. These findings indicate that cytoskeletal alteration can compromise cdk5 activation, regulating p25 formation and suggest that cdk5 inhibitors attenuate apoptosis mediated by cytoskeletal alteration. The present data indicate the potential therapeutic value of drugs that prevent the formation of p25 for the treatment of neurodegenerative disorders.

Keywords: Calcium; Cytoskeleton; Caspases; Calpainsl; Serine-proteases

1. Introduction

In recent years, an enormous effort has been made to clarify the apoptotic pathways involved in neuronal cell death. As a result, it has been hypothesized that intracellular calcium increase is, probably, the first biochemical mediator orchestrating this process [1–3]. The second stage involves, mitochondria modulating this apoptotic route through the release of pro-apoptotic proteins, the best-known being cytochrome c, apoptosis inducing factor (AIF), endonuclease C and SMAC/Diablo [4–8]. All these proteins induce apoptosis through a caspase dependent or independent mechanism. So far, it seems that caspases are the main cysteine proteases involved in the apoptotic process. Fourteen caspases have been identified, but it seems that caspase-3 is the main caspase involved in the process of neuronal cell death. Aside from caspases, other cysteine proteases such as calpains and cathepsins have also been described. Calpain is a calcium dependent cysteine protease implicated in both apoptotic and necrotic processes of neuronal cell death [9,10]. It has been proposed that excessive activation of calpain is involved in Alzheimer's disease and leads to cytoskeletal protein

Abbreviations: AC-DEVD-CHO, Ac-Asp-Glu-Asp-Val-Aldehyde; AC-LEHD-CHO, Ac-Leu-Glu-His-Asp-aldehyde; AIF, apoptosis inducing factor; BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,-*N*′,*N*′-tetraacetic acid tetraacetoxy-methyl ester; CGNs, cerebellar granule neurons; DPQ, 4-dihydro-5[4-(piperindinyl)butoxy]-1(2H)-isoquinoleine; FCS, foetal calf serum; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium]; PARP, poly-APD-ribosil polimerase; PI, propidium iodide; PVDF, polyvinylidene fluoride; TLCK, *N*α-*p*-tosyl-L-lysine chloromethyl ketone; Z-FA-FMK, Z-Phe-Ala fluoromethyl ketone; Z-VEID-FMK, Z-Val-Glu-Ile-Asp-fluoromethylketone

* Corresponding author. Tel.: +34 3 934024531; fax: +34 3 934035982
E-mail address: camins@ub.edu (A. Camins).

[1] The first two authors contributed equally to this work.

[2] Senior co-authors.

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.04.036

breakdown. Furthermore, calpain inhibitors have shown neuroprotective properties in several paradigms such as potassium deprivation in CGNs [9,10]. The last relevant group of proteases that have been identified are cathepsins. These are lysosomal enzymes that are released into the cytoplasm, possibly triggering the neuronal apoptotic process. In support of this are several reports that suggest that lysosomal disturbances contribute to Alzheimer's disease through the accumulation of protein species and the inhibition of axonal/dendritic transport [6,10–15].

In order to understand the mechanism involved in neuronal death, neurotoxins can be used to model neurodegenerative diseases in experimental neuronal cell cultures, such as primary cultures of rat cerebellar granule cells (CGNs) [19,20]. Certain neurotoxins, such as glutamate and kainic acid, are known to induce apoptosis in CGNs by stimulating the ionotropic glutamate receptors and initiating a caspase dependent or independent mechanism (though caspase remains low) [20]. MPP^+ and β-amyloid are neurotoxins used experimentally to model Parkinson's and Alzheimer's diseases, respectively. Both neurotoxins evoke apoptosis in CGNs through the activation of the caspase pathway [16,19,20–23]. Another apoptotic model widely used in CGNs is potassium deprivation, which induced apoptosis through the activation of the intrinsic pathway (mitochondrial), calpains and c-Jun activation and furthermore re-entry into the cell cycle [9,10,17,18]. As neuronal cell death through apoptosis may play a prominent role in neurological disorders, pharmacological compounds that inhibit or attenuate this process may have preventative and therapeutic potential.

Cytoskeletal alteration is a common feature of several neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Unfortunately, the mechanisms involved in apoptosis evoked by such alteration are poorly understood. Thus, experimental models that permit their study must be developed so as to allow the evaluation of new and effective neuroprotective drugs to prevent the progression of neurodegenerative diseases. Several authors, including ourselves, have already reported that the colchicine-induced death of CGNs is an apoptotic process [24–27]. The execution of apoptosis in this model is generally mediated through the release of cytochrome c and caspase-3 activation [25,28]. However, a caspase-independent mechanism, involving the release of apoptosis inducing factor (AIF) [29] has also been demonstrated. It has also been demonstrated that caspase inhibitors only delay apoptosis mediated by colchicine, they do not prevent it [25,26]. These data are in agreement with other studies that have suggested that caspase inhibition is not sufficient to achieve satisfactory neuroprotection [17,19].

The aim of the present study was, therefore to study in-depth the potential apoptotic pathways involved in cytoskeletal alterations mediated by colchicine-induced apoptosis in CGNs. To this end, we evaluated inhibitors of several cysteine proteases, namely caspases, calpains and cathepsins that are well known modulators of the apoptotic process [30,31]. We demonstrated that the alteration in the expression of cdk5 leading to formation of p25 is involved in the regulation of apoptosis in neurons induced by cytoskeleton damage.

2. Materials and methods

2.1. Materials

Pharmacological agents used in this study were as follows: Z-Phe-Ala fluoromethyl ketone (Z-FA-FMK) and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) were from Bachem AG (Bubendorf, Switzerland). PD150606, 4-dihydro-5[4-(piperindinyl)butoxy]-1(2H)-isoquinoleine (DPQ), NU1025 and 1,5-isoquinolinediol were from Calbiochem. Cell culture media and foetal calf serum (FCS) were obtained from GIBCO (Life Technologies, Paisley, U.K.). Kenpaullone, indirubin, AC-DEVD-CHO, Z-VEID-FMK, AC-LEHD-CHO, BAPTA-AM, propidium iodide, Mowiol® 4-88, Triton X-100, enzymes and cell culture salts were from Sigma Chemical Co. (St. Louis, MO, U.S.A). Other chemical reagents were of analytical quality and purchased from Panreac Química (Barcelona, Spain).

2.2. Cell culture

Primary cultures of cerebellar granule cells (CGNs) were prepared from 7-day-old Sprague–Dawley rat pups (from Animal Handling Facilities, University of Barcelone, Spain) as described elsewhere (Verdaguer et al. [19]). Cerebella, freed of meninges, were trypsinized and treated with DNAase. Cell density in solution was adjusted to 8.0×105 cells/mL and cells were then plated on poly-L-lysine-coated plates at a density of 3.2×105 cells/cm^2. Cultures were grown in Eagle's basal medium (BME) containing 10% FCS, 2 mM L-glutamine, 0.1 mg/ml gentamicin and 25 mM KCl. Cytosine arabinoside (10 μM) was added 16–18 h after plating in order to inhibit the growth of non-neuronal cells. Cultures prepared using this method were enriched in granule neurons by more than 95%, assessed routinely by counting GFAP positive cells.

2.3. Treatment of CGNs and viability studies

Experimental treatment of the CGNs was after 7–10 days in vitro. The inhibitory drugs tested were added to the medium, at precise concentrations, 24 h before the addition of 1 μM colchicine. All measures were made after 24 h of colchicine addition.

To assess the loss of cell viability, we used the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] method. MTT was added to cells at a final concentration of 250 μM and incubated for 1 h to allow the reduction of MTT to produce a dark blue formazan product. Media were

removed, and cells were dissolved in dimethylsulfoxide. The production of formazan was measured by absorbance change at 595 nm using a microplate reader (BioRad Laboratories, CA, U.S.A.). Viability results are expressed as percentage relative to the control non-treated cells. The absorbance of non-treated cells was assumed to be 100%.

2.4. Analysis of DNA fragmentation by flow cytometry

To measure apoptosis cells were collected from the culture plates with a pipette and washed with PBS. Flow cytometry test were performed using an Epics XL flow cytometer, adding propidium iodide (PI, 10 μg/ml) 30 min beforehand. The instrument was set up using the standard configuration: the sample was excited using as a standard 488 nm air-cooled argon-ion laser at 15 mW power. Forward scatter (FSC), side scatter (SSC) and red (620 nm) fluorescence for PI were acquired. Optical alignment was based on the optimized signal from 10 nm fluorescent beads (Immunocheck, Epics Division). Time was used as a control of the stability of the instrument red fluorescence was projected on a 1024 monoparametrical histogram. Aggregates were excluded gating single cells by their area versus peak fluorescence signal. Results are shown as percentage of apoptotic cells versus total cells (10,000 cells) counted by the cytometer in each sample.

2.5. Detection of apoptotic nuclei by propidium iodide staining

PI staining was used to detect morphological evidence of apoptosis. CGNs were grown on glass cover slips and after colchicine treatment both alone and in the presence of the different drugs tested in the present study. After the treatment, cells were fixed in 4% paraformaldehyde/phosphate buffered saline solution (PBS), at pH 7.4 for 1 h at room temperature. After washing with PBS, they were incubated for 3 min with a solution of PI in PBS (10 μg/ml). Cover slips were mounted in Mowiol® 4-88. Stained cells were visualized under UV illumination using a 20× objective (NIKON Eclipse 2000T) and their digitized images were captured.

Apoptotic cells could be identified from their shrunken, brightly fluorescent, apoptotic nuclei and condensed chromatin compared to non-apoptotic cells. Apoptotic cells were scored by counting at least 500 cells from six different fields for each sample in three different experiments. Results are shown as a percentage of apoptotic cells in front of total cells in each field.

2.6. Western-blot analysis

Aliquots of cell homogenate, containing 30 μg of protein per sample were analyzed by Western-blot. In brief, samples were placed in sample buffer (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue), and denatured by boiling at 95–100 °C for 5 min and then separated by electrophoresis on 10% acrylamide gels. Afterwards, proteins were transferred to polyvinylidene fluoride (PVDF) sheets (ImmobilonTM-P, Millipore Corp., Bedford, MA) using a transblot apparatus (BioRad). Membranes were blocked overnight with 5% non-fat milk dissolved in TBS-T buffer (Tris 50 mM; NaCl 1.5%; Tween 20, 0.05%, pH 7.5). They were then incubated with primary monoclonal antibodies against cdk5 (sc-173) and p35/p25 (sc-820) (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). After 90 min, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG antibody (Amersham Corp., Arlington Heights, IL). Immunoreactive protein was visualized using a chemiluminescence-based detection kit following the manufacturer protocol (ECL kit; Amersham Corp.). Routinely, protein load was monitored using phenol red staining of the blot membrane or immunodetection of α-tubulin.

2.7. Immunocytochemistry against cdk5

CGNs were grown on sterile glass slides. After treatment, cells were washed twice in PBS and fixed in 4% paraformaldehyde/PBS, pH 7, 4 for 1h at room temperature. Cells were pre-incubated for 30 min with PBS containing 0.3% Triton X-100 and 30% normal horse serum at room temperature. After blocking, cells were incubated with a antibody against cdk5 (1:400, sc-173, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 0.3% Triton X-100 and 3% normal horse serum, overnight at 4 °C. Cells were then washed extensively and incubated with fluorescent secondary antibody for 1 h at room temperature. Coverslips were thoroughly washed and mounted in Mowiol® 4-88 and immunosignal analysis was performed using fluorescence microscopy at 100x magnification (NIKON Eclipse 2000T).

2.8. Statistical analysis

Data are given as mean ± S.E.M. from at least quadruplicate experiments across four to six independent cultures. Data were analysed in all experiments by ANOVAs followed by post hoc Tukey–Kramer multiple comparisons tests and p-values lower that 0.05 were considered significant.

3. Results

3.1. Caspase activation pathway in colchicine-induced apoptosis in CGNs

The effectiveness of selective caspase-3 and -6 inhibitors (concentration range 10–100 μM) were studied.

AC-DEVD-CHO, a selective caspase-3 inhibitor, did not show any neuroprotective effect. It decreased neither cell death nor apoptosis induced by colchicine in CGNs (Figs. 1A and 4). Notably, pretreatment with Z-VEID-FMK, a selective caspase-6 inhibitor also failed to decrease colchicine-induced cell death/apoptosis in the cultures or attenuate on neurotoxicity as measured by nuclear cell counting and flow cytometry. Finally, we evaluated the effects of the upstream of caspase-9-selective inhibitor, AC-LEHD-CHO (ranging from 10 to 100 μM) (Figs. 1A and 4). Pretreatment of CGNs with this peptide was also ineffective in preventing of cell death and apoptosis. On the other hand, Z-VAD-FMK (100 μM) was able to prevent the neurotoxicity induced by colchicine measured by MTT, flow cytometry and condensed nuclei counting (Figs. 1A and 4).

3.2. Calpain activation in colchicine-induced apoptosis in CGNs

To explore the possible involvement of calpain-regulated pathway in colchicine-induced neural death, we also

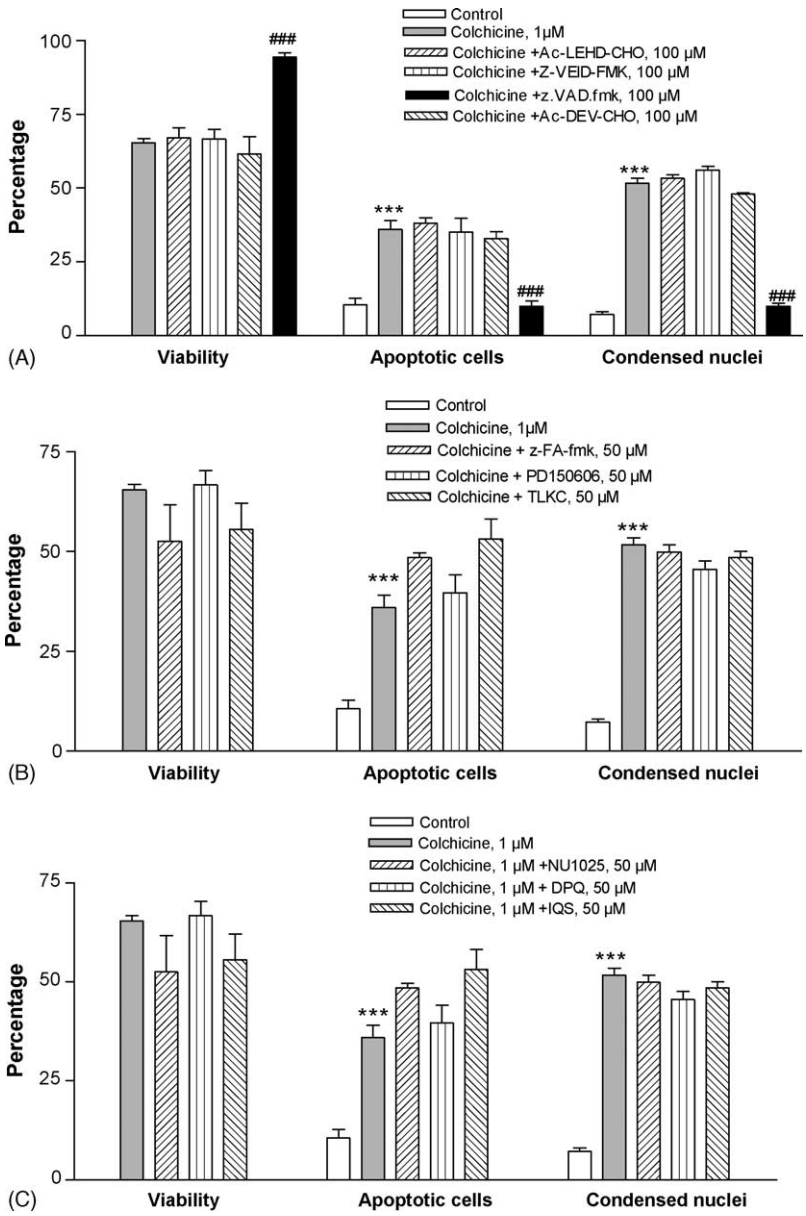

Fig. 1. Bar chart showing the effects of the different compounds tested on 1 μM colchicine-induced toxicity in CGNs exposed for 24 h. Viability is based on MTT assays, apoptotic cells are based on Flow cytometry analyses and condensed nuclei counting by propidium iodide staining under fluorescence illumination. The nuclei were counted on a fluorescence microscope, distinguishing normal nuclei from the condensed ones following the criteria stated in the Section 2. Each point is the mean ± S.E.M. of four wells of five to six different cultures. (A) Lack of activity for caspase-3, -6 and -9 only Z-VAD-FMK (100 μM) prevents 1 μM colchicine-induced apoptosis in CGNs. (B) Bar chart showing the percentage of apoptotic cells in the presence of protease inhibitor, cathepsin inhibitor, serine-proteases inhibitor and calpain inhibitor. (C) Analyses of different parameters in the presence of PARP-1 inhibitors. When necessary, statistical analyses was carried out using the one-way ANOVAs followed by Tukey's tests: $^{***}p < 0.001$ vs. control; $^{\#\#\#}p < 0.001$ vs. colchicine.

examined the effects of the synthetic cell-permeable calpain inhibitor, PD150606 [38,39]. Pretreatment with PD150606 (concentration range 1–60 μM) was not able to increase the cell viability of CGNs exposed to colchicine, as shown by MTT assays. In addition, no difference was seen in rates of cell apoptosis between CGNs treated with PD150606 plus colchicine (Figs. 1A and 4).

3.3. Implication of serine-proteases in colchicine-induced apoptosis in CGNs

Previous studies have revealed a caspase-independent apoptotic pathway mediated by colchicine in neuronal preparations [28]. Thus, we evaluated the involvement of other non-caspase proteases using TLCK a serine-protease inhibitor, relatively specific to trypsin-like enzymes. Pre-treatment of CGNs with TLCK (ranging from 10 to 50 μM) did not increase neuronal survival as measured by MTT (Figs. 1A and 4). Likewise, TLCK did not attenuate the apoptotic process mediated by cytoskeletal disruption (Figs. 1A and 4).

3.4. Involvement of cathepsins in colchicine-induced apoptosis

Previous studies have suggested that lysosomal proteases act as proapoptotic mediators in several cell systems [6,12,13]. Z-FA-FMK, a cathepsin-inhibitor, specific to cathepsin D and L, was not able to prevent the reduction of MTT activity caused by colchicine. Furthermore, treatment of neurons with this compound (ranging 10–50 μM) did not block colchicine-mediated nuclear condensation of

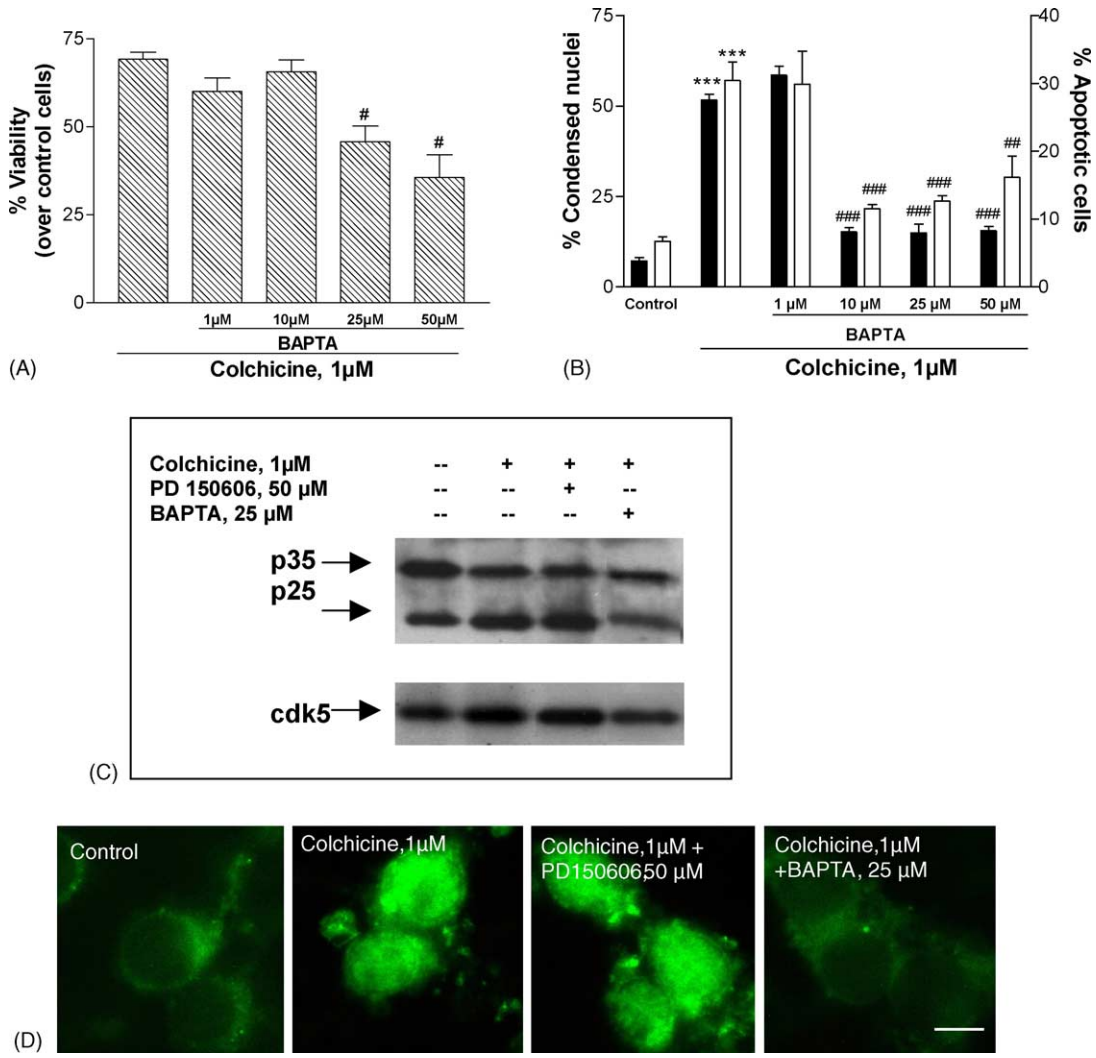

Fig. 2. Bar chart showing the effects of BAPTA on 1 μM colchicine-induced toxicity in CGNs exposed for 24 h. (A) MTT assay showing a lack of protection by different concentrations of BAPTA all failing to protect against toxicity. (B) Flow cytometry analysis of 1 μM colchicine-induced apoptosis (open bars) and nuclei condensation (filled bars) in permeabilised CGNs exposed to colchicine for 24 h in the absence or presence of BAPTA. (C) Western-blot analysis of the levels of cdk5 and p35/p25 in CGNs control cultures and those treated with colchicine (1 μM) in the absence or the presence of BAPTA (25 μM) and PD 150606 (50 μM) Similar results were obtained in three independent experiments. (D) cdk5 immunoreactiviy in CGNs, after colchicine-induced toxicity and loss of staining in the presence of 25 μM BAPTA. PD 150606 did not exert any effect on the fluorescence signal. Statistical analyses was carried out using one-way ANOVAs followed by Tukey's tests $^{***}p < 0.001$ vs. Control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. colchicine 1 μM. Calibration bar, 10 μm.

CGNs nor apoptotic peak formation as measured by flow cytometry (Figs. 1A and 4).

3.5. Effects of PARP-1 inhibitors in colchicine-induced apoptosis

Poly (ADP-ribose) polymerase-1 is a nuclear enzyme that is primarily activated by DNA damage. Recent studies also suggest that PARP-1 is involved in a caspase-independent apoptotic process, by regulating the release of apoptosis-inducing factor (AIF). We, thus, evaluated in the present study the role of several PARP-1 inhibitors in colchicine-induced apoptosis in CGNs. Three selective PARP-1 inhibitors DPQ, NU 1025 and ISQ were all ineffective in preventing the decrease in MTT values and preventing from colchicine-induced apoptosis (ranging 1–50 μM) (Fig. 1C). These data suggest that PARP-1 is not involved in the apoptotic pathway of cytoskeletal alteration in our experimental conditions (Figs. 1C and 4).

3.6. Effects of BAPTA-AM on colchicine-induced apoptosis in CGNs

It is well known that intracellular calcium is a key modulator of the apoptotic process [6]. Therefore, we were carried out several experiments to evaluate the effects of BAPTA-AM, an intracellular calcium chelator (concentration range 1–50 μM) on colchicine-induced apoptosis. In our experimental conditions, BAPTA-AM was not able to restore the decline in MTT reduction induced by colchicine ($63 \pm 3\%$, Fig. 2A). Indeed, high concentrations of BAPTA-AM potentiated the neuronal damage mediated by colchicine. On the other hand, when we evaluated the antiapoptotic effects of BAPTA-AM, through flow cytometry and nuclear cell counting, we found an antiapoptotic effect at low concentrations (10 and 25 μM). Colchicine produced an increase in apoptosis measured by flow cytometry of $33.5 \pm 1.6\%$, compared to control values, but in the presence of 10, 25 and 50 μM of BAPTA-AM, apoptosis was reduced up to 11.5 ± 0.6; 12.7 ± 0.8; 16.1 ± 3.1, respectively (Figs. 2A and 4). The nuclear morphology of neurons was also evaluated using the fluorescent PI. While cells in the control condition were characterized by regular and round nuclei, treatment with colchicine increased the number of condensed nuclei up to 45–50%. BAPTA-AM (10 μM) reduced this colchicine-induced nuclear damage, and nuclear morphology was similar to that in the control cells (Figs. 2A and 4). These data suggest that intracellular calcium levels modulate the apoptotic process mediated by colchicine, and are relevant to neuronal survival.

Likewise, it has been hypothesized that intracellular calcium is implicated in p35/25 breakdown, promoting cdk5 activation and initiating an apoptotic process. As expected colchicine produced an increase in cdk5 protein levels as shown in Western-blot assays and immunocytochemistry analysis. As shown in Fig. 2C and D, BAPTA-AM 25 μM produces change in p25/p35 ratio, preventing the increase in the p25 fragment. Because calpain activation is involved in p25/p35 breakdown, we evaluated the effects of PD150606 on this pathway; as showed in Fig. 2C and D this compound did not change the protein profile studies, according to neurotoxicity studies (Figs. 1B and 4).

3.7. Neuroprotective properties of kenpaullone, a GSK-3β/CDK5 inhibitor, against colchicine-induced neurotoxicity in CGNs

To determine more thoroughly the involvement of cdk5/p25 in the neurotoxic effects of colchicine, we used a specific GSK-3β/cdk5 inhibitor, kenpaullone. Kenpaullone was added to CGNs cultures (1–50 μM) in the presence of colchicine 1 μM. As shown in Fig. 3A, kenpaullone significantly increases neuronal survival from 60 to 80%. To examine the antiapoptotic properties of kenpaullone, we evaluated characteristic features of apoptosis, such as nuclear condensation and fragmentation in colchicine treated CGNs. While colchicine induced a 50% increase in apoptotic cells, kenpaullone 25 μM, significantly decreased this percentage to 17% (Figs. 3A and 4). Morphological apoptotic evaluation of CGNs using PI staining and fluorescent microscopy revealed a percentage of 9% in control samples that significantly increased to 55% in colchicine treated neurons. Kenpaullone 25 μM significantly reduced the number of condensed nuclei until 17% (Figs. 3A and 4).

Further experiments were carried out to evaluate the inhibition of the expression of cdk5 by kenpaullone after colchicine treatment in CGNs. Western-blot data (Fig. 3C) revealed that kenpaullone 25 μM decreases the expression of cdk5 and prevents p35 cleavage to p25 after treatment with 1 μM colchicine. Immunocytochemical data corroborate this evidence of inhibition of cdk5 expression (Fig. 3B and D).

We also examined the antiapoptotic effects of induribin sulphonic-acid on apoptotic features, cdk5 expression and p35/p25 breakdown. Indirubin (10 to 50 μM) showed clear antiapoptotic properties in CGNs in this experimental model of cytoskeletal alteration (Fig. 4B). As expected, cdk5 inhibitor reduced cdk5 expression and p35/p25 activation as indicated by Western-blot and immunocytochemistry experiments (Fig. 3C and D).

4. Discussion

The aim of the present study was to characterize the potential intracellular signalling cascade involved in neuronal death after cytoskeletal alteration. Rat CGNs can be induced to undergo apoptosis after treatment with colchicine and this form of cell death has been described as apoptotic. Caspases are involved in this apoptotic model of cytoskeletal alteration. Previously, we have demonstrated

that the pan-caspase inhibitor Z-VAD-fmk prevents apoptosis mediated by colchicine [24,26]. In previous studies, it has also been demonstrated that colchicine-induced neurotoxicity is mediated through mitochondrial alterations following the release of cytochrome c and activation of caspase-3 [17,22,29]. However, the upstream and downstream events leading to apoptosis through colchicine exposure remained relatively unknown. In the present work, we demonstrated that, although the caspase pathway is activated, selective inhibitors of caspases-3, -6 and -9 (AC-DEVD-CHO, Z-VEID-FMK and AC-LEHD-CHO, respectively) failed to provide neuroprotection against colchicine-induced cell death. These results could be interpreted as suggesting that selective inhibition of a specific caspase involved in the intrinsic apoptotic pathway is not sufficient to afford neuroprotection in this apoptotic model [25,26,29]. They are in agreement with Mitsui et al. [40], who demonstrated the lack of neuroprotective properties of caspase-3 inhibitor, Ac-DEVD-CHO and -9 inhibitor, Ac-LEHD-CHO, on colchicine-induced apoptosis in superior cervical ganglion cells.

On the other hand, several authors, including ourselves have demonstrated that the pan-caspase inhibitor Z-VAD-FMK shows neuroprotective properties in the face of colchicine-induced apoptosis. The most likely explanation is that, in this apoptotic model of cytoskeletal alteration, it is necessary to completely inhibit caspases involved in the intrinsic pathway in order to afford real and efficient neuroprotection. Furthermore, others studies have demonstrated that Z-VAD-FMK inhibits others proteases that could be involved in neuronal apoptosis such as calpains and cathepsins [32–34]. Therefore, in this apoptotic model,

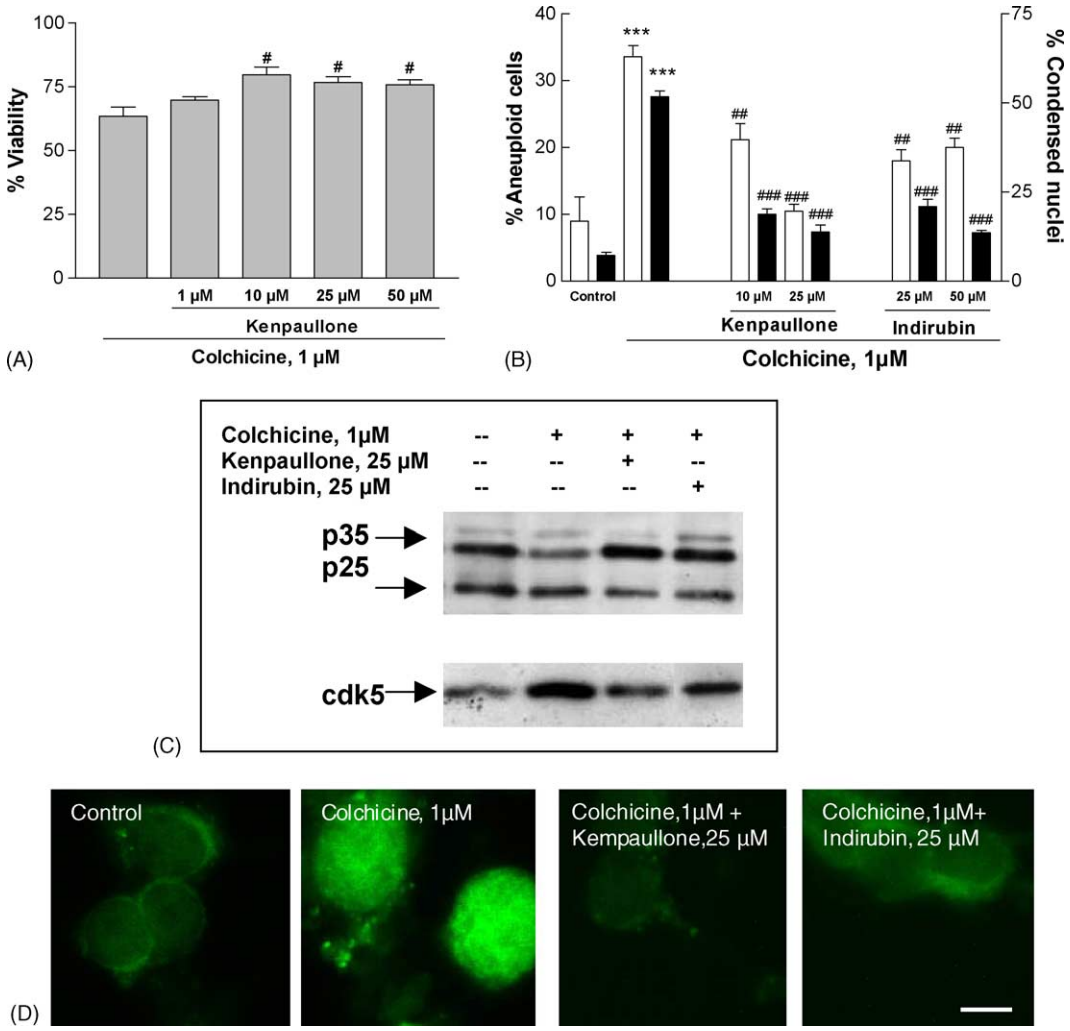

Fig. 3. Bar chart showing the effects of kenpaullone and indirubine on 1 μM colchicine-induced toxicity in CGNs exposed for 24 h. (A) MTT assay showing significant neuroprotection by different concentrations of kenpaullone. (B) Flow cytometry analysis of 1 μM colchicine-induced apoptosis (open bars) and nuclei condensation (filled bars) in permeabilised CGNs exposed to colchicine for 24 h in the absence or presence of kenpaullone and indirubine. (C) Western-blot analysis of the levels of cdk5 and p35/p25 in CGNs control cultures and those treated with colchicine (1 μM) in the absence or presence of kenpaullone and indirubine. Similar results were obtained in three independent experiments. (D) cdk5 immunoreactiviy in CGNs, after colchicine-induced toxicity and loss of staining in the presence of kenpaullone and indirubine. Calibration bar, 10 μm. Statistical analyses were carried out using one-way ANOVAs followed by Tukey's tests $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. colchicine 1 μM. Calibration bar, 10 μm.

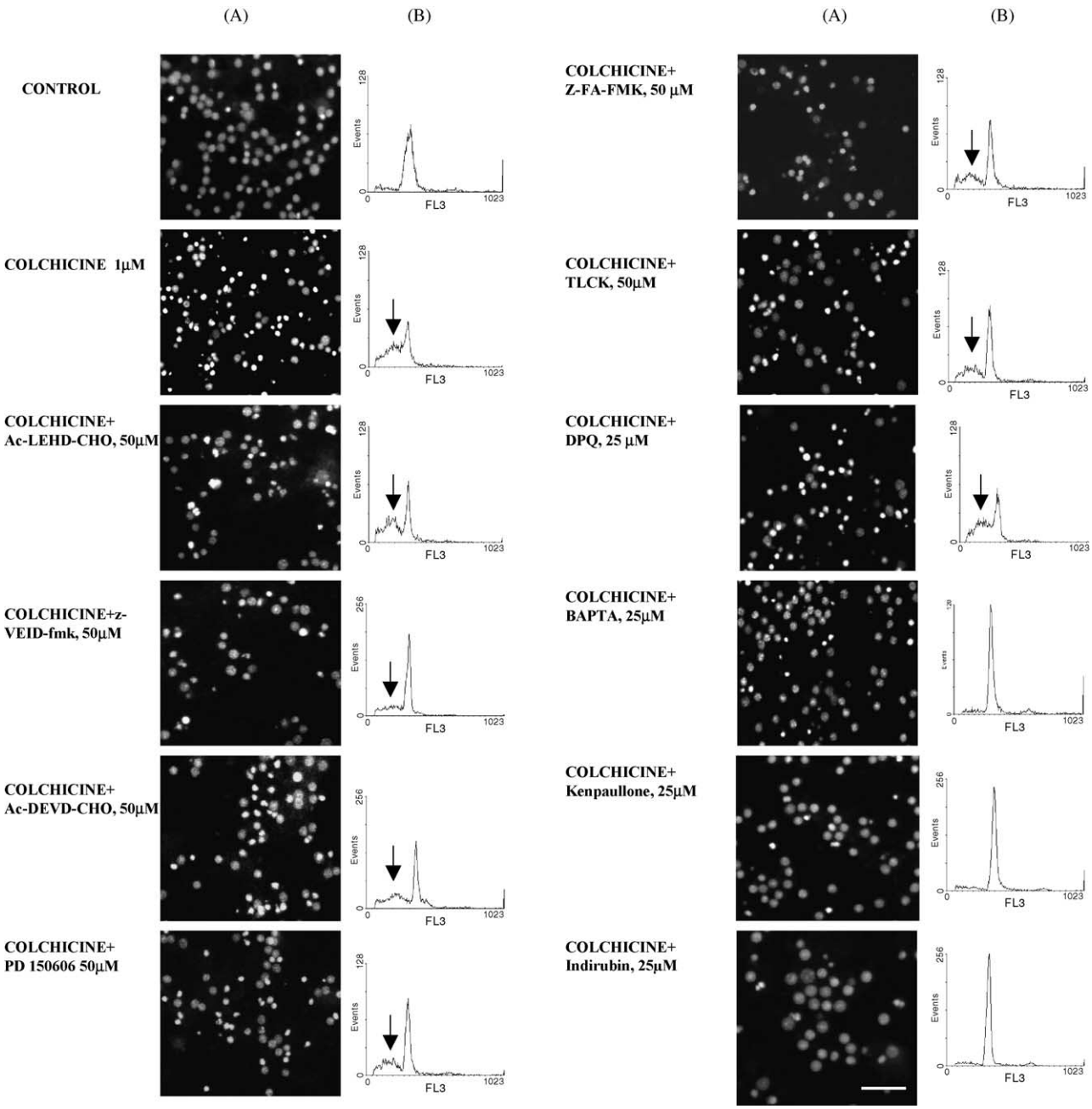

Fig. 4. Effects of the various treatments assayed on CGN. (A) Representative fluorescence photomicrographs showing chromatin condensation in permeabilised CGNs. Calibration bar, 10 μM. (B) Representative flow cytometric histograms of PI-stained cells after those treatments indicated to the left. Arrows indicate apoptotic nuclei.

it may be necessary to simultaneously inhibit several cysteine proteases in order to obtain suitable neuroprotection. However, it is important to emphasize that in previous studies, Volbracht et al. have demonstrated that Z-VAD-FMK only offers partial neuroprotection against colchicine, delaying more than preventing the apoptotic process. This result is in agreement with other authors which demonstrated that caspase inhibitors delay apoptosis mediated by MPP^+ and 6-hydroxydopamine, both experimental models of Parkinson's disease [20].

Although it is well known that the apoptotic process is regulated by cysteine aspartate proteases (caspases), other studies suggest the implication of others cysteine proteases in the apoptotic process, e.g. calpains [9,10,33,34]. We, therefore, looked for neuroprotective effects of PD151606, a selective calpain inhibitor, but without success. These data indicate that calpain-specific inhibition is not sufficient to prevent apoptosis mediated by colchicine in CGNs. Although in previous studies, Mitsui et al. demonstrated that TLCK, a serine proteases inhibitor, attenuates colchicine-mediated apoptosis, in our experimental conditions we were not able to detect any neuroprotective effect. The same authors also demonstrated that the administration of Z-VAD-FMK

in conjunction with TLCK offers major neuroprotection [41]. Stefanis et al. [13] demonstrated that inhibitors of trypsin-like serine proteases show neuroprotective effects in neuronal cell preparations after treatment with DNA damaging agents. However, our results indicate that trypsin-like serine proteases, in our experimental conditions, are not involved in colchicine apoptosis.

Several studies suggest the implication of lysosomal proteases, such as cathepsins, as mediators of the apoptotic process in several cellular systems [6,11–13]. In the search for the apoptotic route involved in apoptosis induced by cytoskeletal alterations, we evaluated the effect of Z-FA-FMK, a cathepsin inhibitor. The process of programmed cell death regulated by lysosomes is known as autophagy [6,11–13]. Our experimental data indicate that cathepsin inhibition is not sufficient to prevent apoptosis induced by cytoskeletal alteration, but more studies are necessary to evaluate the potential role of autophagy in apoptosis induced by cytoskeletal alteration in CGNs.

DNA neuronal damage and cell cycle progression are possible triggers to apoptosis. PARP-1 is an enzyme involved in DNA repair and it is dramatically activated when DNA is damaged [37]. Previous studies suggest that PARP-1 activation is involved in the regulation of AIF [38]. In the present study, PARP-1 inhibitors did not prevent apoptosis mediated by colchicine, indicating, in agreement with others, that colchicine-induced apoptosis in neurons progresses through a mechanism independent of DNA damage.

Re-entry to the cell cycle is regulated by the activation of cyclin-dependent kinases, specifically cdk4/cdk2. It is well known that neurons are quiescent cells that do not proliferate [41]. Therefore, in neurons an attempt to re-enter the cell cycle is associated with the apoptotic process by the activation of the transcription factor E2F-1. Recently, Kruman et al. [42] demonstrated that the presence of DNA synthesis in mature neurons is associated with apoptosis through different neurotoxic stimuli known to damage DNA. Our previous results, using the excitotoxin kainic acid and potassium deprivation in CGNs, are in agreement with the hypothesis correlating cell cycle re-entry with the apoptotic process. Meanwhile, Kruman et al. demonstrated that staurosporine and colchicine, which do not damage DNA, did no induce re-entry to the cell cycle [42,43]. Thus, neither PARP-1 activation nor cell cycle activation is involved in apoptosis induced by cytoskeletal alteration.

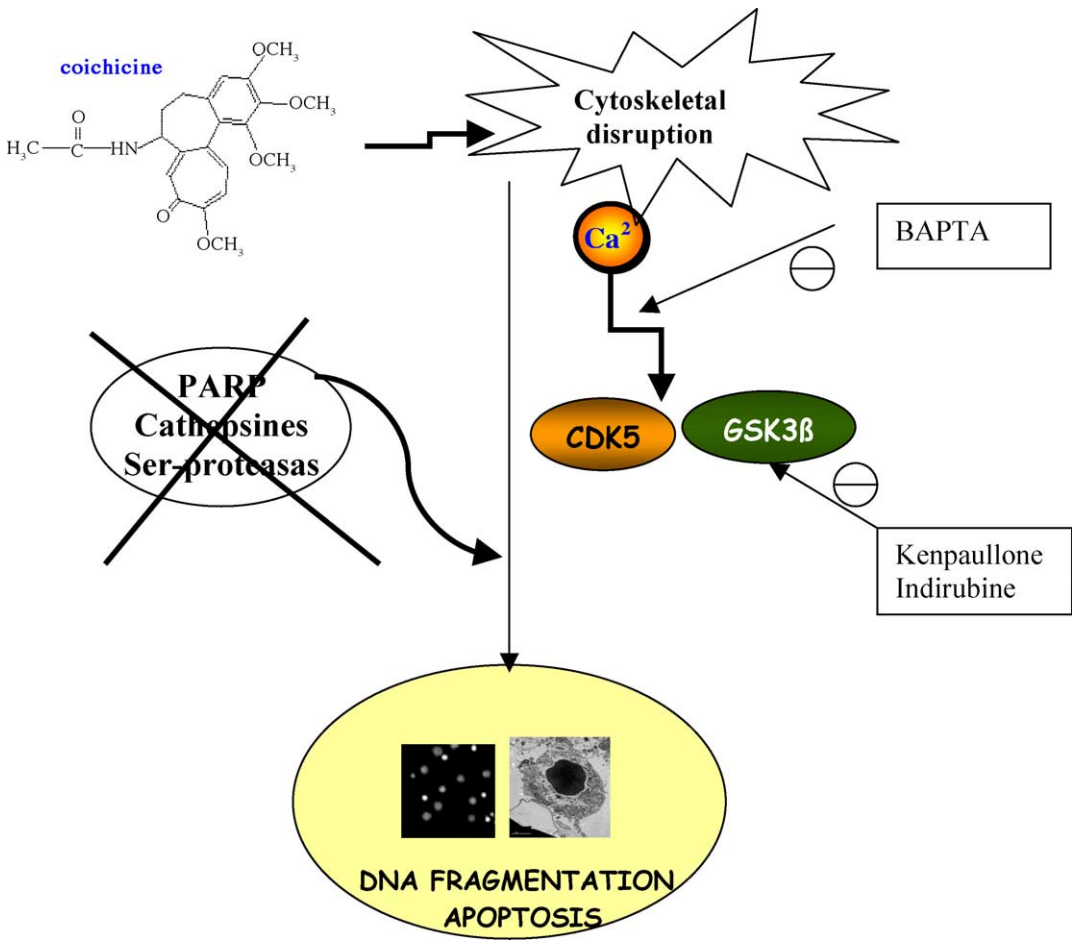

Fig. 5. Proposed mechanism whereby colchicine promotes neuronal apoptosis linked to cdk5 and GSK-3β activation.

Previous studies in our laboratory show that neurotoxic stimuli that are not able to evoke re-entry to the cell cycle in neurons, induced an increase in the expression of cdk5. It has been demonstrated that cdk5 is a kinase that is not involved in the control of the cell cycle, but is implicated in the process of tau hyperphosphorylation and the cytoskeleton destructuration [44–48]. Recent studies demonstrated that cdk5/p25 is involved in the process of neuronal cell death in different paradigms such as excitotoxicity, oxidative stress and staurosporine-induced apoptosis. In fact, colchicine induces apoptosis through microtubule destabilization and tau dephosphorylation [49,50]. It is well known that the process of tau phosphorylation and dephosphorylation is a process regulated by cdk5, GSK-3β and phosphatase activities [45–51].

The activation of the cdk5/p25 route is dependent on calcium concentration. Therefore, an important factor is the role of intracellular calcium in colchicine-induced apoptosis in CGNs. In the present study, the effects on colchicine-induced apoptosis of different concentrations of BAPTA-AM, an intracellular calcium chelator, were evaluated. Previous studies have demonstrated that, depending on concentration, BAPTA-AM can produced contradictory effects in neuronal cell preparations, being either: neuroprotective or neurotoxic [35,36]. Furthermore, it is well known that, calcium is necessary for neuronal survival, but that excessive calcium chelating can be neurotoxic. Cell viability results indicate that BAPTA-AM was not able to prevent the decrease off MTT induced by colchicine in CGNs. However, this compound significantly attenuated apoptosis, indicating calcium is involved in the modulation of apoptotic process in CGNs. Likewise, BAPTA-AM, prevents the cleavage of p35 to p25. This is a process in which calpains are implicated, but we did not find any neuroprotective effect, neither in viability nor apoptosis, of PD 150606, suggesting that there are additional regulators of p25 formation [48,49]. This hypothesis is in accordance with previous studies where calcium was shown to be responsible for the formation of p25 [46].

Nevertheless, in the present study, we demonstrated that kenpaullone and induribin, cdk5/p25 pathway inhibitors [52,53], were very effective in inhibiting colchicine-induced apoptosis in CGNs. We have previously demonstrated that flavopiridol and roscovitine, two well known cell-cycle inhibitors, show antiapoptotic properties in this model [26]. However, because these compounds also inhibit transcription, we decided to use others most specific to cdk5/GSK-3β in this study in order to confirm our hypotheses.

To conclude, the data presented in the present study provide strong evidence that cdk5 regulate the apoptotic process in this model in CGNs (Fig. 5). Our data highlight the key role of the cdk5/p25 pathway in this apoptotic model and, in agreement with our previous studies, we propose that cyclin dependent kinases play an important role in the neuronal apoptotic process.

Acknowledgements

The authors would like to acknowledge to Maria Teresa Iglesias for precious technical assistance. The excellent secretarial support of Ms. Mar Morales is greatly appreciated. We thank the Language Advice Service of the University of Barcelona for revising the manuscript. This study was supported by SAF2002-00790 (FEDER), FISS G03/137, FISS G03/167, PI04/1300, Interdisziplinäre Zentrum für klinishe Forschung (IZKF) Leipzig at the Faculty of Medicine of the University of Leipzig. Ester Verdaguer is a recipient of a Humboldt Research Fellowships.

References

[1] Friedlander RM. Apoptosis and caspases in neurodegenerative diseases. N Engl J Med 2003;348:1365–75.
[2] Roth KA. Caspases, apoptosis, and Alzheimer Disease: Causation, correlation, and confusion.
[3] Waldmeier PC, Tatton WG. Interrupting apoptosis in neurodegenerative disease: potential for effective therapy? DDT 2004;9:210–8.
[4] Hickey MA, Chesselet MF. Apoptosis in Huntington's disease. Prog Neuropsychopharmacol Biol Psychiatry 2003;27:255–65.
[5] Kerr JFR. History of the events leading to the formulation of the apoptosis concept. Toxicology 2002;181–182:471–4.
[6] Stefanis L. Caspase-dependent and -independent neuronal death: two distinct pathways to neuronal injury. Neuroscientist 2005; 11:50–62.
[7] Van Loo G, Saelens X, van Gurp M, MacFarlane M, Martin SJ, Vandenabeele P. The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. Cell Death Differ 2002; 9:1031–42.
[8] Nicotera P, Leist M. Energy supply and the shape of death in neurons and lymphoid cells. Cell Death Differ 1997;4:435–42.
[9] Nath R, Raser KJ, Mcginnis K, Nadimpalli R, Stafford D, Wang KKW. Effects of ICE-like protease and calpain inhibitors on neuronal apoptosis. Neuroreport 1996;8:249–55.
[10] Nath R, Raser KJ, Stafford D, Hajimohammadreza I, Posner A, Allen H, et al. 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 1996;319:683–90.
[11] Rideout HJ, Stefanis L. Caspase inhibition: a potential therapeutic strategy in neurological diseases. Histol Histopathol 2001;16: 895–908.
[12] Lang-Rollin IC, Rideout HJ, Noticewala M, Stefanis L. Mechanisms of caspase-independent neuronal death: energy depletion and free radical generation. J Neurosci 2003;23:11015–25.
[13] Stefanis L, Troy CM, Qi H, Greene LA. Inhibitors of trypsin-like serinee proteases inhibit processing of the caspase Nedd-2 and protect PC12 cells and sympathetic neurons from death evoked by withdrawal of trophic support. J Neurochem 1997;69:1425–37.
[14] Shimohama S. Apoptosis in Alzheimer's disease. An update. Apoptosis 2000;5:9–16.
[15] Maccioni RB, Munoz JP, Barbeito L. The molecular bases of Alzheimer's disease and other neurodegenerative disorders. Arch Med Res 2001;32:367–81.
[16] Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. Science 2003;302:819–22.
[17] Nicotera P. Apoptosis and age-related disorders: role of caspase-dependent and caspase-independent pathways. Toxicol Lett 2002; 127:189–95.

[18] Cribbs DH, Poon WW, Rissman RA, Blurton-Jones M. Caspase-mediated degeneration in Alzheimer's disease. Am J Pathol 2004; 165:353–5.
[19] Verdaguer E, Garcia-Jorda E, Jimenez A, Stranges A, Sureda FX, Canudas AM, et al. Kainic acid-induced neuronal cell death in cerebellar granule cells is not prevented by caspase inhibitors. Br J Pharmacol 2002;135:1297–307.
[20] Gonzalez-Polo RA, Soler G, Fuentes JM. MPP+: mechanism for its toxicity in cerebellar granule cells. Mol Neurobiol 2004;30:253–64.
[21] Gonzalez-Polo RA, Soler G, Rodriguez-Martin A, Moran JM, Fuentes JM. Protection against MPP+ neurotoxicity in cerebellar granule cells by antioxidants. Cell Biol Int 2004;28:373–80.
[22] Leist M, Volbracht C, Fava E, Nicotera P. 1-Methyl-4-phenylpyridinium induces autocrine excitotoxicity, protease activation, and neuronal apoptosis. Mol Pharmacol 1998;54:789–801.
[23] Allen JW, Eldadah BA, Huang X, Knoblach SM, Faden AI. Multiple caspases are involved in beta-amyloid-induced neuronal apoptosis. J Neurosci Res 2001;65:45–53.
[24] Jorda EG, Verdaguer E, Morano A, Jimenez A, Canudas AM, Camins A, et al. Lithium prevents colchicine-induced apoptosis in rat cerebellar granule neurons. Bipolar Disord 2004;6:144–9.
[25] Dare E, Gorman AM, Ahlbom E, Gotz M, Momoi T, Ceccatelli S. Apoptotic morphology does not always require caspase activity in rat cerebellar granule neurons. Neurotoxicol Res 2001;3:501–14.
[26] Jorda EG, Verdaguer E, Canudas AM, Jimenez A, Bruna A, Caelles C, et al. Neuroprotective action of flavopiridol, a cyclin-dependent kinase inhibitor, in colchicine-induced apoptosis. Neuropharmacology 2003;45:672–83.
[27] Volbracht C, Leist M, Kolb SA, Nicotera P. Apoptosis in caspase-inhibited neurons. Mol Med 2001;7:36–48.
[28] Volbracht C, Leist M, Nicotera P. ATP controls neuronal apoptosis triggered by microtubule breakdown or potassium deprivation. Mol Med 1999;5:477–89.
[29] Fonfria E, Dare E, Benelli M, Sunol C, Ceccatelli S. Translocation of apoptosis-inducing factor in cerebellar granule cells exponed to neurotoxic agents inducing oxidative stress. Eur J Neurosci 2002;16:2013–6.
[30] Bendiske J, Bahr BA. Lysosomal activation is a compensatory response against protein accumulation and associated synaptopathogenesis an approach for slowing Alzheimer disease? J Neuropathol Exp Neurol 2003;62:451–63.
[31] Gan L, Ye S, Chu A, Anton K, Yi S, Vincent VA, et al. Identification of cathepsin B as a mediator of neuronal death induced by Abeta-activated microglial cells using a functional genomics approach. J Biol Chem 2004;279:5565–72.
[32] Waterhouse NJ, Finucane DM, Green DR, Elce JS, Kumar S, Alnemri ES, et al. Calpain activation is upstream of caspases in radiation-induced apoptosis. Cell Death Differ 1998;5:1051–61.
[33] Ray SK, Hogan EL, Banik NL. Calpain in the pathophysiology of spinal cord injury: neuroprotection with calpain inhibitors. Brain Res Rev 2003;42:169–85.
[34] Ray SK, Banik NL. Calpain and its involvement in the pathophysiology of CNS injuries and diseases: therapeutic potential of calpain inhibitors for prevention of neurodegeneration. Curr Drug Targets CNS Neurol Disord 2003;173–89.
[35] Tseng WP, Lin-Shiau SY. Calcium-activated NO production plays a role in neuronal death induced by beta-bungarotoxin in primary cultures of cerebellar granular neurons. Naunyn Schmiedebergs Arch Pharmacol 2003;367:451–61.
[36] Han KS, Kang HJ, Kim EY, Yoon WJ, Sohn S, Kwon HJ, et al. 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid induces caspase-mediated apoptosis and reactive oxygen species-mediated necrosis in cultured cortical neurons. J Neurochem 2001;78:230–9.
[37] Hong SJ, Dawson TM, Dawson VL. Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. Trends Pharmacol Sci 2004;25:259–64.
[38] Cregan SP, Dawson VL, Slack RS. Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 2004;23:2785–96.
[39] Kristensen BW, Noer H, Gramsbergen JB, Zimmer J, Noraberg J. Colchicine induces apoptosis in organotypic hippocampal slice cultures. Brain Res 2003;964:264–78.
[40] Mitsui C, Sakai K, Ninomiya T, Koike T. Involvement of TLCK-sensitive serinee protease in colchicine-induced cell death of sympathetic neurons in culture. J Neurosci Res 2001;66:601–11.
[41] Verdaguer E, Jimenez A, Canudas AM, Jorda EG, Sureda FX, Pallas M, et al. Inhibition of cell cycle pathway by flavopiridol promotes survival of cerebellar granule cells after an excitotoxic treatment. J Pharmacol Exp Ther 2004;308:609–16.
[42] Kruman II, Wersto RP, Cardozo-Pelaez F, Smilenov L, Chan SL, Chrest FJ, et al. Cell cycle activation linked to neuronal cell death initiated by DNA damage. Neuron 2004;41:549–61.
[43] Kruman II. Why do neurons enter the cell cycle? Cell Cycle 2004;3:769–73.
[44] Canu N, Dus L, Barbato C, Ciotti MT, Brancolini C, Rinaldi AM, et al. Tau cleavage and dephosphorylation in cerebellar granule neurons undergoing apoptosis. J Neurosci 1998;18:7061–74.
[45] Rametti A, Esclaire F, Yardin C, Terro F. Linking alterations in tau phosphorylation and cleavage during neuronal apoptosis. J Biol Chem 2004;279:54518–28.
[46] Lee MS, Kwon YT, Li M, Peng J, Friedlander, RM, Tsai LH. Neurotoxicity indeces cleavage of p35 to p25 by calpain. 2000;405:360–4.
[47] Zheng YL, Kesavapany S, Gravell M, Hamilton RS, Schubert M, Amin N, et al. A Cdk5 inhibitory peptide reduces tau hyperphosphorylation and apoptosis in neurons. EMBO J 2005;24:209–20.
[48] Cruz JC, Tsai LH. Cdk5 deregulation in the pathogenesis of Alzheimer's disease. Trends Mol Med 2004;10:452–8.
[49] Cheung ZH, Ip NY. Cdk5: mediator of neuronal death and survival. Neurosci Lett 2004;361:47–51.
[50] Morfini G, Szebenyi G, Brown H, Pant HC, Pigino G, DeBoer S, et al. A novel CDK5-dependent pathway for regulating GSK3 activity and kinesin-driven motility in neurons. EMBO J 2004;23:2235–45.
[51] Zambrano CA, Egaña JT, Núñez MT, Maccioni RB, Gonzalez-Billaut C. Oxidative stress promotes τ dephosphorylation in neuronal cells: roles of cdk5 and PP1. Free Rad Biol Med 2004;36: 1393–402.
[52] Damiens E, Baratte B, Marie D, Eisenbrand G, Meijer L. Anti-mitotic properties of indirubin-3′-monoxime, a CDK/GSK-3 inhibitor: induction of endoreplication following prophase arrest. Oncogene 2001;28:3786–97.
[53] Hoessel R, Leclerc S, Endicott JA, Nobel ME, Lawrie A, Tunnah P, et al. Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. Nat Cell Biol 1999; 1:60–7.