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