Biochemical and Biophysical Research Communications 371 (2008) 127–131

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Calbindin-D28K prevents drug-induced dopaminergic neuronal death by inhibiting caspase and calpain activity

Won-Seok Choi, Eunhee Lee, Junghyun Lim, Young J. Oh *

Department of Biology and Protein Network Research Center, Yonsei University, 134 Shincheondong Seodaemoonku, College of Science, Seoul 120-749, Republic of Korea

ARTICLE INFO

Article history:
Received 2 April 2008
Available online 14 April 2008

Keywords:
Calbindin-D28K
MPP+
Staurosporine
Calcium
Caspase
Neuronal death

ABSTRACT

Calbindin-D28K protects against apoptotic and necrotic cell death; these effects have been attributed to its ability to buffer calcium. In this study, we investigated the mechanisms underlying the neuroprotective effects of calbindin-D28K in staurosporine (STS)-induced apoptosis and 1-methyl-4-phenylpyridinium (MPP^+)-induced necrosis. Treatment of the dopaminergic neuronal cell line MN9D with STS or MPP^+ induced cell death that was associated with increased levels of free intracellular calcium. However, only MPP^+-induced death was inhibited by co-treatment of the cells with a calcium chelator or a sodium/calcium antiporter inhibitor. Overexpression of calbindin-D28K prevented MPP^+-induced MN9D cell death, which occurs in the absence of any detectable caspase activation. These pro-survival effects of calbindin-D28K were associated with the inhibition of calcium-mediated calpain activation, as determined by processing of Bax. Overexpression of calbindin-D28K also blocked STS-induced MN9D death. However, this effect was accompanied by the inhibition of capase-3 cleavage, poly(ADP-ribose)polymerase cleavage, and caspase activity. These findings suggest that calbindin-D28K protects against both types of cell death by inhibiting caspase- or calcium-mediated death signaling pathway.

Calcium has been implicated in the control of various neuronal processes, including excitability, transmission, differentiation, survival, and death [1]. Many classes of neurons express calcium-binding proteins that have a common structural domain known as the EF-hand [2]. Calcium-binding proteins maintain calcium homeostasis by buffering excessive intracellular levels of free calcium $[Ca^{2+}]_i$. Calbindin-D28K belongs to the EF-hand family of these proteins [3] and is abundant throughout the central nervous system [4]. Several reports have indicated that calbindin-D28K has neuroprotective effects in ischemic and glutamate toxicity models, primarily due to its ability to chelate calcium [5–7]. Recently, biochemical evidence has suggested that in osteoblasts, calbindin-D28K binds directly to caspase-3 and inhibits its activity. Therefore, it is possible that calbindin-D28K is able to prevent cell death through more than one mechanism [8].

Previously, we demonstrated that staurosporine (STS) induces caspase-dependent neuronal cell death that is morphologically and biochemically typical of apoptosis [9]. In contrast, the dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+) induces caspase-independent and calpain-dependent necrotic cell death in the MN9D dopaminergic neuronal cell line, and also in primary cultures of mesencephalic and cortical neurons [10–13]. In order to test the hypothesis that calbindin-D28K intervenes in more than one death pathway to promote neuronal survival, we investigated the protective effects of calbindin-D28K in MN9D dopaminergic neuronal cells exposed to calcium-dependent or calcium-independent death stimuli. In this study, we provide evidence that calbindin-D28K protects neuronal cells against STS-induced (calcium-independent) and MPP^+-induced (calcium-dependent) death via two separate mechanisms.

Materials and methods

Cell culture. MN9D cells stably overexpressing calbindin-D28K (MN9D/Calbindin) and the expression vector alone (MN9D/Neo) have been established [14]. Neither the MN9D parental cells nor the MN9D/Neo cells expressed detectable levels of endogenous calbindin-D28K as determined by immunoblot analysis [14]. Cells were seeded at a density of 2×10^4 cells in 48-well plates (Costar) or 1×10^6 cells in P-100 dishes (Corning) that had been coated with 25 μg/ml poly-D-lysine. Cultures were then maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and 500 μg/ml G418 (Life Technologies; complete culture medium, CCM) for 3 days in an atmosphere of 10% CO_2 at 37 °C. Cells were subsequently switched to serum-free N2 medium [15] or serum-free N2 medium containing 1 μM staurosporine (STS; Sigma) or 50 μM 1-methyl-4-phenylpyridinium (MPP^+; RBI). In a subset of experiments, the serum-free medium also contained 40 μM BAPTA, 4 μM CGP 37157, or 50 μM calpeptin (all from Calbiochem).

MTT reduction assay. Following drug treatment, cells grown in 48-well plates were subjected to a MTT reduction assay to assess cell survival. MTT reduction assays were performed as previously described [10]. Cell survival after each treatment was expressed as a percent of survival in the untreated control (100% survival).

* Corresponding author. Fax: +82 2 312 5657.
E-mail address: yjoh@yonsei.ac.kr (Y.J. Oh).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.04.020

Cytosolic calcium measurement. Intracellular calcium levels were measured using Fura-2 fluorescence videomicroscopy. Cells grown in 35 mm glass-bottom dishes coated with poly-D-lysine were incubated with 5 μM of the cytosolic calcium indicator, Fura-2/AM at room temperature for 30 min and with HEPES-buffered salt solution for an additional 30 min. Cells were imaged at room temperature using a Leica TCS NT system confocal microscope (Leica, Heidelberg, Germany). Images of the Fura-2 (ex: 340–380 nm; em: 510 nm) ratio were acquired with a LSM 510 camera (Carl Zeiss).

Immunoblot analysis. Following drug treatment, cells were washed with ice-cold PBS and lysed for 10 min in a buffer containing 50 mM Tris (pH 7.0), 2 mM EDTA, 1% Triton X-100, 2 mM PMSF, and 10 μg/ml leupeptin and aprotinin. Cellular lysates were centrifuged at 13,000*g* for 15 min at 4 °C. Proteins in the supernatant were measured using a Bio-Rad protein assay reagent. Forty micrograms of protein from each sample were separated by a 10–12.5% SDS–PAGE. The proteins were then electroblotted onto PVDF membrane (Bio-Rad). The membranes were then probed with the following primary antibodies: rabbit anti-calbindin-D28K (1:5000; Swant, Bellinzona, Switzerland), rabbit anti-cleavage products of caspase-3 (1:1000; Cell Signaling), mouse monoclonal anti-poly-(ADP-ribose)-polymerase (1:5000; Enzyme System Products), and rabbit anti-Bax antibody (1:3000; a generous gift from Dr. J.C. Reed). Following washing and incubation of the membranes with the appropriate HRP-conjugated secondary antibodies (1:3,000; Amersham Bioscience), specific bands were detected using enhanced chemiluminescence (ECL; Amersham Bioscience).

Fluorogenic caspase substrate assay. Cellular lysates were prepared in 50 mM Tris (pH 7.0), 2 mM EDTA, and 1.0% Triton X-100. Lysates (10 μg) were incubated with 25 μM Ac-DEVD-AMC (Calbiochem) for 1 h at 37 °C in buffer containing 100 mM HEPES (pH 7.4), 10% sucrose, 5 mM dithiothreitol, and 0.1% CHAPS. The production of the fluorescent cleavage product was monitored at Ex380/Em460 nm using a FL 600 plate reader (Bio-Tek). In separate experiments, the direct interaction between calbindin-D28K and caspase-3 was investigated by incubating 5 μg of purified caspase-3 ±2 μg of purified mouse calbindin-D28K in buffer containing 0.5% Nonidet P-40, 20 mM HEPES (pH 7.4), 100 mM NaCl, and 20 mM dithiothreitol. Reactions were incubated at 37 °C for 1 h before assaying the cleavage products.

Statistics. Data were analyzed using one-way ANOVA and a *post hoc* Student's *t* test. All values are reported as means ± SEM for the indicated number of experiments. Values of $p < 0.05$ were considered significant.

Results

We previously demonstrated that MN9D dopaminergic neuronal cells undergo caspase-dependent apoptosis within 24 h of exposure to STS, whereas treatment with the dopaminergic neurotoxin, MPP^+ induces caspase-independent and calpain-dependent necrotic cell death within 48 h [9–13]. In the present study, we

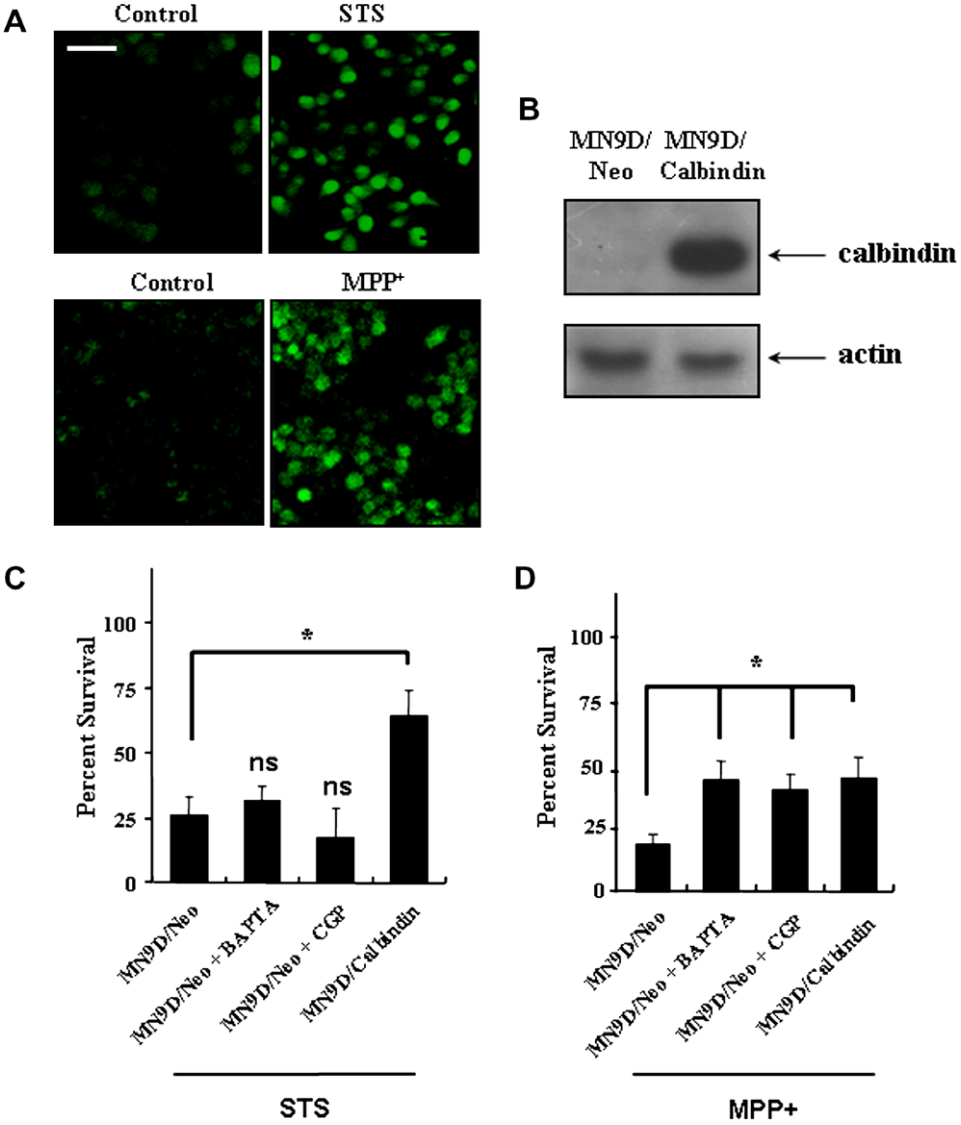

Fig. 1. Overexpression of calbindin prevents drug-induced cell death. (A) Fura-2 staining of MN9D cells treated with 1 μM STS for 1 h or with 50 μM MPP^+ for 24 h. Scale bar, 50 μm. (B) Immunoblot analysis of calbindin-D28K in MN9D cells stably transfected with chick calbindin-D28K (MN9D/Calbindin) or vector (MN9D/Neo). (C, D) MTT reduction assay of MN9D/Neo and MN9D/Calbindin cells following (C) 24 h of STS exposure or (D) 48 h of MPP^+ exposure, in the presence or absence of 40 μM BAPTA or 4 μM CGP37157. Values were expressed as a percentage relative to the untreated control (100%). All experiments were repeated 3–5 times in triplicate. $^*p < 0.05$; ns, not significant.