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Differential effect of calmodulin antagonists on MG132-induced mitochondrial dysfunction and cell death in PC12 cells

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Abstract

Defects in proteasome function have been suggested to be involved in the pathogenesis of neurodegenerative diseases. We examined the effect of calmodulin antagonists on proteasome inhibitor-induced mitochondrial dysfunction and cell viability loss in undifferentiated PC12 cells. Caspase inhibitors (z-IETD.fmk, z-LEHD.fmk and z-DQMD.fmk) and antioxidants attenuated cell death and decrease in GSH contents in PC12 cells treated with 20 μM MG132, a proteasome inhibitor. Calmodulin antagonists (trifluoperazine, W-7 and calmidazolium) had a differential inhibitory effect on the MG132-induced cell death and GSH depletion depending on concentration with a maximal inhibitory effect at 0.5–1 μM. Addition of trifluoperazine and W-7 reduced the MG132-induced nuclear damage, loss of the mitochondrial transmembrane potential followed by cytochrome *c* release, formation of reactive oxygen species and elevation of intracellular Ca^{2+} levels in PC12 cells. Calmodulin antagonists at 5 μM exhibited a cytotoxic effect on PC12 cells but attenuated the cytotoxicity of MG132. The results suggest that the toxicity of MG132 on PC12 cells is mediated by activation of caspase-8, -9 and -3. Trifluoperazine and W-7 at the concentrations of 0.5–1 μM may attenuate the MG132-induced viability loss in PC12 cells by suppressing change in the mitochondrial membrane permeability and by lowering of the intracellular Ca^{2+} levels as well as calmodulin inhibition.

Keywords: Calmodulin antagonists; MG132; Mitochondrial membrane permeability; Cell injury; PC12 cells

1. Introduction

The ubiquitin/proteasome pathway, responsible for mediating the majority of intracellular proteolysis, plays a crucial role in the regulation of many normal cellular processes, including the cell cycle, differentiation and apoptosis [2,23]. Defects in proteasome function have been suggested to be involved in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease [26]. Proteasome inhibitors have been shown to induce apoptosis in neuronal cells characterized by nuclear fragmentation, loss of mitochondrial membrane potential, cytochrome *c* release and caspase activation [11,18,27]. In contrast, proteasome inhibitors lactacystin and AcLLNaI induce apoptotic death in human glioma cells by a mitochondria-independent mechanism [13]. In addition, it is uncertain whether the cytotoxicity of MG132 is mediated by elevation of the intracellular Ca^{2+} levels [14,31].

Disruption of intracellular calcium homeostasis and defects in mitochondrial function mediate neuronal cell death [6,36]. Elevation of intracellular Ca^{2+} levels leads to the production of reactive oxygen species (ROS) and nitrogen species [5]. Ca^{2+} overload may excessively activate Ca^{2+}/calmodulin-dependent pathways and leads to irreversible cell damage [8]. Calmodulin antagonists such as W-7 and trifluoperazine inhibit cell death due to 1-methyl-4-phenylpyridinium (MPP^+) or veratridine-induced depolarization [15,32]. Trifluoperazine as an inhibitor of the mitochondrial permeability transition is demonstrated to reduce oxidative stress-induced cell death by suppressing the cytochrome *c* release and mitochondrial depolarization

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[29,30]. Meanwhile, phenothiazines, including trifluoperazine, show a strong cytotoxicity and antiproliferative activity against neuronal cells and leukemic cells [7,39].

MG132, which effectively blocks the proteolytic activity of 26S proteasome complex, causes apoptotic cell death through formation of ROS [3,27,38]. Although calmodulin antagonist such as trifluoperazine is demonstrated to reduce oxidative cell injury, the effect on mitochondrial damage and apoptotic cell death due to MG132 has not been elucidated. The aim of the present study was to explore the effect of calmodulin antagonists (trifluoperazine, W-7 and calmidazolium) on the MG132-induced mitochondrial dysfunction and cell death in PC12 cells. This study examined the effect of calmodulin antagonists on mitochondrial damage and subsequent cytochrome *c* release, changes in intracellular Ca^{2+} levels, formation of ROS and cellular GSH contents.

2. Materials and methods

2.1. Chemicals

TiterTACS™ colorimetric apoptosis detection kit was purchased from Trevigen, Inc. (Gaithersburg, MD, USA) Quantikine® M rat/mouse cytochrome *c* assay kit was from R&D systems (Minneapolis, MN, USA) and ApoAlert™ CPP32/caspase-3 assay kit was from CLONTECH Laboratories Inc. (Palo Alto, CA, USA). z-Asp-(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone (z-DQMD.fmk) and z-Ile-Glu-(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone (z-IETD.fmk) were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). Z-Leu-Leu-Leu-al (MG132), trifluoperazine, *N*-(6-aminohexyl)-5-chloro-1-naphalenesulfonamide (W-7), calmidazolium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), 2′,7′-dichlorofluorescin diacetate ($DCFH_2$-DA), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Protein concentration was determined by the method of Bradford according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

2.2. Cell culture

Rat PC12 cells (adrenal gland; pheochromocytoma) were cultured in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin according to the manual of the cell line bank. Cells were washed with RPMI medium containing 1% FBS 24 h before experiments and replated onto the 96- and 24-well plates.

2.3. Assessment of cell viability

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [20]. PC12 cells (4×10^4) were treated with MG132 for 24 h at 37 °C. The medium (200 μl) was incubated with 10 μg/ml of 10 mg/ml MTT solution for 2 h. Culture medium was removed and dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA, USA). Cell viability was expressed as a percentage of value in control cultures.

2.4. Morphological observation of nuclear change

PC12 cells (1×10^6) were treated with MG132 for 24 h at 37 °C and the nuclear morphological change was assessed using the Hoechst dye 33258 [22]. Cells were incubated with 1 μg/ml Hoechst 33258 for 3 min at room temperature and nuclei were visualized using an Olympus Microscope with a WU excitation filter (Tokyo, Japan).

2.5. Measurement of apoptosis in cells

Apoptosis was assessed by measuring the DNA fragmentation, which occurs following the activation of endonucleases. PC12 cells (1×10^5) were treated with MG132 for 24 h at 37 °C, washed with phosphate-buffered saline (PBS) and fixed with formaldehyde solution. Nucleotide (dNTP) was incorporated at the 3′-ends of DNA fragments using terminal deoxynucleotidyl transferase (TdT) and the nucleotide was detected using a streptavidine-horseradish peroxidase and TACS-Sapphire according to TiterTACS protocol. Data were expressed as absorbance at 450 nm.

2.6. Flow cytometric measurement of mitochondrial transmembrane potential

Changes in the mitochondrial transmembrane potential during the MG132-induced apoptosis in PC12 cells were quantified by flow cytometry with the cationic lipophilic dye $DiOC_6(3)$ [10]. Cells (1×10^6) were treated with MG132 for 24 h at 37 °C, $DiOC_6(3)$ (40 nM) was added to the medium and cells incubated for 15 min at 37 °C. After centrifugation at $412 \times g$ for 10 min, the supernatants were removed and the pellets were resuspended in PBS containing 0.5 mM EDTA. For analysis, a FACScan cytofluorometer (Becton Dickinson, San Jose, CA, USA) with argon laser excitation at 501 nm was used to assess 10,000 cells from each sample.

2.7. Measurement of cytochrome c release

The release of cytochrome *c* from mitochondria into the cytosol was assessed by using a solid phase, enzyme-linked immunosorbent assay for the detection of cytochrome *c*. PC12 cells (5×10^5) harvested by centrifugation at $412 \times g$ for 10 min were washed twice with PBS and suspended in buffer (in mM): sucrose 250, KCl 10, $MgCl_2$ 1.5, EDTA 1,