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Econazole attenuates cytotoxicity of 1-methyl-4-phenylpyridinium by suppressing mitochondrial membrane permeability transition

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Abstract

Defects in mitochondrial function have been shown to participate in the induction of neuronal cell injury. The effect of econazole against the cytotoxicity of 1-methyl-4-phenylpyridinium (MPP^+) in differentiated PC12 cells was assessed in relation to the mitochondrial membrane permeability changes. Treatment of PC12 cells with MPP^+ resulted in the nuclear damage, decrease in the mitochondrial transmembrane potential, cytosolic accumulation of cytochrome *c*, activation of caspase-3, increase in the formation of reactive oxygen species (ROS) and depletion of GSH. Econazole (0.25–2.5 μM) inhibited the cytotoxicity of MPP^+ or rotenone. The addition of econazole (0.5 μM) significantly attenuated the MPP^+-induced mitochondrial damage, elevation of intracellular Ca^{2+} level and cell death. However, because of the cytotoxicity, econazole at 5 μM did not attenuate the toxicity of MPP^+. The results show that econazole at the low concentrations may reduce the MPP^+-induced viability loss in PC12 cells by suppressing the mitochondrial permeability transition, leading to activation of caspase-3 and the elevation of intracellular Ca^{2+} levels, which are associated with the increased formation of ROS and depletion of GSH.

Keywords: 1-Methyl-4-phenylpyridinium; Econazole; PC12 cells; Mitochondrial membrane permeability; Cell injury

1. Introduction

The membrane permeability transition of mitochondria is known as a central event in the course of a variety of toxic and oxidative forms of cell injury as well as apoptosis [9,29]. Neuronal cell death due to 1-methyl-4-phenylpyridinium (MPP^+) is mediated by opening of the mitochondrial permeability transition pore, releases of Ca^{2+} and cytochrome *c* and activation of caspases [4,19]. Formation of the mitochondrial permeability transition due to exposure of MPP^+ seems to be associated with increased oxidative stress [17]. Infusion of MPP^+ into the brains of mice and rats increases the formations of lipid peroxides and hydroxyl radicals in the striatum [25,31]. MPP^+ is demonstrated to stimulate the displacement of dopamine from vesicular storage sites to the cytoplasm, which further induces an oxidation of dopamine to liberate free radicals and dopamine quinone that are involved in mitochondrial damage and cell death [20].

It has been shown that econazole, an imidazole anti-fungal drug, causes the mitochondrial Ca^{2+} loading, protein synthesis inhibition and cell death by inducing elevation of intracellular Ca^{2+} levels and depletion of Ca^{2+} from endoplasmic reticulum [33,34]. However, econazole inhibits the bradykinin- or arachidonic acid-induced increase in the intracellular Ca^{2+} levels by blocking Ca^{2+} influx [2,35]. In contrast to these reports, econazole does not attenuate the hydrogen peroxide-induced Ca^{2+} influx [3] and not reduce the elevation of intracellular Ca^{2+} levels due to the apoptosis-inducing betulinic acid [7].

Compared to the studies against the cytotoxic action of econazole, the protective action has been rarely reported. Econazole inhibits the cytolytic granulysin-induced mitochondrial damage and apoptotic cell death by suppressing the elevation of intracellular Ca^{2+} levels [27]. Ethanol is known to cause cell death by inducing mitochondrial damage and cytochrome *c*, which is accompanied by the activation of caspase-3 and 9 [13]. An imidazole anti-fungal ketoconazole protects gastric mucosa against the ethanol-induced depletion of non-protein sulfhydryl groups [1].

The effect of econazole against cell injury as well as changes in the intracellular Ca^{2+} levels remains to be uncertain. Furthermore, the effect of econazole on the MPP^+-induced mitochondrial damage and cell death has not been elucidated. Therefore, the present study was performed to assess the effect

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of econazole on differentiated PC12 cells against the toxicity of MPP^+ in relation to the mitochondrial permeability transition.

2. Materials and methods

2.1. Materials

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), anti-cytochrome *c* (A-8) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), horseradish peroxidase-conjugated anti-mouse IgG was from EMD-Calbiochem. Co. (La Jolla, CA, USA), SuperSignal® West Pico chemiluminescence substrate was from PIERCE Biotechnology Inc. (Rockford, IL, USA), ApoAlert™ CPP32/caspase-3 assay kit was from CLONTECH Laboratories Inc. (Palo Alto, CA, USA) and Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (Mn-TBAP) was from OXIS International Inc. (Portland, OR, USA). Econazole, 1-methyl-4-phenylpyridinium, rotenone, catalase (from bovine liver; 10,000–25,000 units/mg protein), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), 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), phenylmethylsulfonylfluoride (PMSF) and other chemicals were purchased from Sigma–Aldrich Co. (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 as described in the manual of the cell line bank. Cells were differentiated by treating with 100 ng/ml 7S nerve growth factor for 9 days [37]. Cells were washed with RPMI medium containing 1% FBS 24 h before experiments and replated onto the 96- and 24-well plates.

2.3. Cell viability assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [23]. PC12 cells (4×10^4 cells/200 μl) were treated with MPP^+ for 24 h at 37 °C. The medium was incubated with 10 μl of 10 mg/ml MTT solution for 2 h. After centrifugation at $412 \times g$ for 10 min, the culture medium was removed and 100 μl 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 the value in control cultures.

2.4. Morphological observation of nuclear change

PC12 cells (2×10^5 cells/ml) were treated with MPP^+ for 24 h at 37 °C and the nuclear morphological change was assessed using the Hoechst dye 33258 [26]. Cells were washed 1 ml phosphate-buffered saline (PBS) and incubated with 1 μg/ml Hoechst 33258 for 3 min at room temperature. 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 (4×10^4 cells/200 μl) were treated with MPP^+ for 24 h at 37 °C, washed with PBS and fixed with 3.7% buffered 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 MPP^+-induced apoptosis in PC12 cells were quantified by flow cytometry with the cationic lipophilic dye $DiOC_6(3)$ [14]. Cells (1×10^6 cells/ml) were treated with MPP^+ for 24 h at 37 °C, $DiOC_6(3)$ (40 nM) 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 suspended in 1 ml of 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 kit and Western blot analysis. PC12 cells (5×10^5 cells/ml for ELISA assay and 5×10^6 cells for Western blotting) were harvested by centrifugation at $412 \times g$ for 10 min, washed twice with PBS, resuspended in buffer (in mM): sucrose 250, KCl 10, $MgCl_2$ 1.5, EDTA 1, EGTA 1, dithiothreitol 0.5, PMSF 0.1 and HEPES-KOH 20, pH 7.5 and homogenized further by successive passages through a 26-gauge hypodermic needle. The homogenates were centrifuged at $100{,}000 \times g$ for 30 min and the supernatant was used for analysis of cytochrome *c*. The supernatants were added to the 96-well microplates coated with monoclonal antibody specific for rat/mouse cytochrome *c* that contains cytochrome *c* conjugate. The procedure was performed according to the manufacturer's instructions. Absorbance of samples was measured at 450 nm in a microplate reader. A standard curve was constructed by adding diluted solutions of cytochrome *c* standard, handled like samples, to the microplates coated with monoclonal antibody. The amount was expressed as ng/ml by reference to the standard curve.

For Western blotting, supernatants were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. Samples (30 μg/ml protein) were loaded onto each lane of 15% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked for 2 h in TBS (50 mM Tris–HCl, pH 7.5 and 150 mM NaCl) containing 0.1% Tween 20 and 5% non-fat dried milk. The membranes were labeled with anti-cytochrome *c* (diluted 1:1000 in TBS containing 0.1% Tween 20) for 2 h at room temperature with gentle agitation. After four washes in TBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:2000) for 2 h at room temperature. Protein bands were identified with the enhanced chemiluminescence detection using SuperSignal® West Pico chemiluminescence substrate.

2.8. Measurement of caspase-3 activity

PC12 cells (2×10^6 cells/ml) were treated with MPP^+ for 24 h at 37 °C and caspase-3 activity was determined according to the user's manual for the ApoAlert™ CPP32/Caspase-3 assay kit. The supernatant obtained by a centrifugation of lysed cells was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) and incubated for 1 h at 37 °C. Absorbance of the chromophore *p*-nitroanilide produced was measured at 405 nm. The standard curves were obtained from the absorbances of *p*-nitroanilide standard reagent diluted with cell lysis buffer (up to 20 nM). One unit of the enzyme was defined as the activity producing 1 nmol of *p*-nitroanilide.

2.9. Measurement of intracellular reactive oxygen species (ROS) formation

The dye $DCFH_2$-DA, which is oxidized to fluorescent DCF by hydroperoxides, was used to measure relative levels of cellular peroxides [12]. PC12 cells (4×10^4 cells/200 μl) were treated with MPP^+ for 24 h at 37 °C, washed,