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Chloride channels involve in hydrogen peroxide-induced apoptosis of PC12 cells

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ABSTRACT

Chloride channel activity is one of the critical factors responsible for cell apoptotic volume decrease (AVD). However, the roles of chloride channels in apoptosis have not been fully understood. In the current study, we assessed the role of chloride channels in hydrogen peroxide (H_2O_2)-induced apoptosis of pheochromocytoma cells (PC12). Extracellular application of H_2O_2 activated a chloride current and induced cell volume decrease in a few minutes. Incubation of cells with H_2O_2 elevated significantly the membrane permeability to the DNA dye Hoechst 33258 in 1 h and induced apoptosis of most PC12 cells tested in 24 h. The chloride channel blocker NPPB (5-nitro-2-(3-phenylpropylamino)-benzoate) prevented appearance of H_2O_2-induced high membrane permeability and cell shrinkage, suppressed H_2O_2-activated chloride currents and protected PC12 cells from apoptosis induced by H_2O_2. The results suggest that chloride channels may contribute to H_2O_2-induced apoptosis by ways of elevation of membrane permeability and AVD in PC12 cells.

Introduction

Accumulating evidence has shown that increased apoptosis in the central nervous system is associated with neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and multiple sclerosis, which have a massive progressive loss of different populations of neurons [1]. The pathogenesis of these diseases is associated with oxygen free radicals, which can induce severe cell damage through oxidative stress. The synthesis of reactive oxygen species (ROS) (e.g. O^{2-}, superoxide) can be increased by stress or cerebral ischemia. The PC12 cell, a rat pheochromocytoma cell line, is capable of generating catecholamines, possess physiological and biochemical characteristics of neurons and is widely used in the research of Alzheimer's disease and Parkinson's disease [2]. Hydrogen peroxide (H_2O_2) is a member of the ROS family and frequently used as a toxicant to establish in vitro model of oxidative stress-induced cell damage [3]. Previous studies on the apoptosis of PC12 cells induced by oxidative damage mostly focus on mitochondrial membrane depolarization [4], cytochrome c release [5], caspase activation [6,7], cell and nucleus condensation [8], DNA fragmentation and signaling pathways [9,10]. However, the relationship between chloride channels and apoptosis has not been well-explored. In the current study, we assessed the role of chloride channels in H_2O_2-induced apoptosis of PC12 cells.

Materials and methods

Cell culture and preparation for measurements of cell volume and currents. PC12 cells were routinely grown in RPMI 1640 medium (GIBCO) with 15% newborn calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin in the humidified atmosphere with 5% CO_2 and 95% O_2 at 37 °C. Cell were collected, resuspended, plated on 22-mm round coverslips and incubated for 30 min before volume regulation experiments or current recordings.

Solutions and chemicals. The isotonic bath solution contained (in mM): 70 NaCl, 0.5 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 140 D-mannitol. The pipette solution consisted of (in mM): 70 *N*-methyl-D-glucamine chloride (NMDG-Cl), 1.2 $MgCl_2$, 10 HEPES, 1 EGTA, 140 D-mannitol and 2 ATP. The pH of bath and pipette solutions was adjusted to 7.4 and 7.25. 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB, Sigma) was dissolved in DMSO (100 mmol/L) and diluted to final concentrations with isotonic solutions or culture media.

Whole cell current recording. Whole cell currents were recorded with 5–10 MΩ pipettes and an EPC-7 patch clamp amplifier (HEKA, Germany) at 20–24 °C. Membrane potential was held at the Cl^- equilibrium potential (0 mV) and stepped to 0, ±40 and ±80 mV for 200 ms repeatedly, with 4s intervals between pulses. Using a laboratory interface (CED 1401, Cambridge, UK), voltages and currents were recorded in computers. Currents were measured at 10 ms after onset of voltage steps.

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Volume measurements. Cell images were captured every 60s by a CCD camera (Mono CCD625, Leica) at 20–24 °C and analyzed with Scion software (Scion Corporation). Cell volume was computed from cell diameters. Experiments were conducted in six groups: control, 100, 300 and 600 μmol/L H_2O_2, NPPB alone and mixture of H_2O_2 and NPPB. In the control, cells were bathed in isotonic solution for 65 min. In groups of H_2O_2 or NPPB alone, cells were incubated in control solutions for 5 min and in H_2O_2 or 100 μmol/L NPPB for 60 min. In mixture group, cells were bathed in control solution for 5 min, in 100 μmol/L NPPB solution for 5 min and in the solution with 300 μmol/L H_2O_2 plus 100 μmol/L NPPB for 60 min.

Apoptosis monitored by flow cytometry. Cells cultured for 24 h were collected, washed with phosphate-buffered saline (PBS), fixed in 70% ethanol at −20 °C for 30 min, washed with PBS and incubated in RNase A (50 μg/ml in PBS) at 37 °C for 30 min, stained with propidium iodide (50 μg/ml) for 15 min and analyzed by the flow cytometry. For each sample, 10,000–20,000 cells were analyzed. The percentage of apoptosis cells was quantified from DNA histograms.

Nuclear staining analysis by Hoechst 33258. Cells were washed with PBS, stained with DNA dye Hoechst 33258 (5 μg/ml) for 10 min and rinsed with PBS. Fluorescence images were acquired and analyzed. Apoptotic cells or cells with high membrane permeability displayed bright blue nuclei, while normal cells emitted only weak blue fluorescence.

Statistics. Data were expressed as the mean ± standard error (with *n* indicating the number of observations). An analysis of variance (ANOVA) was used to analyze the data and differences were considered significant at $P < 0.05$.

Results

Chloride channel blocker NPPB attenuates H_2O_2-induced apoptosis in PC12 cells

PCl2 cell apoptosis was evaluated by flow cytometry (Fig. 1). In the control, apoptotic cells were only 3.0 ± 0.2% in the population ($n = 4$). However, exposure to 300 μmol/L H_2O_2 for 24 h increased apoptotic rate greatly to 70.2 ± 3.2% ($n = 4$, $P < 0.01$). In the culture medium containing H_2O_2 and the chloride channel blocker NPPB (100 μmol/L), the apoptosis rate dropped to 40.5 ± 4.0% ($n = 4$, $P < 0.01$). Treatments with 100 μmol/L NPPB alone did not affect apoptotic rate significantly (4.9 ± 1.2%, $n = 4$; $P > 0.05$, compared with control).

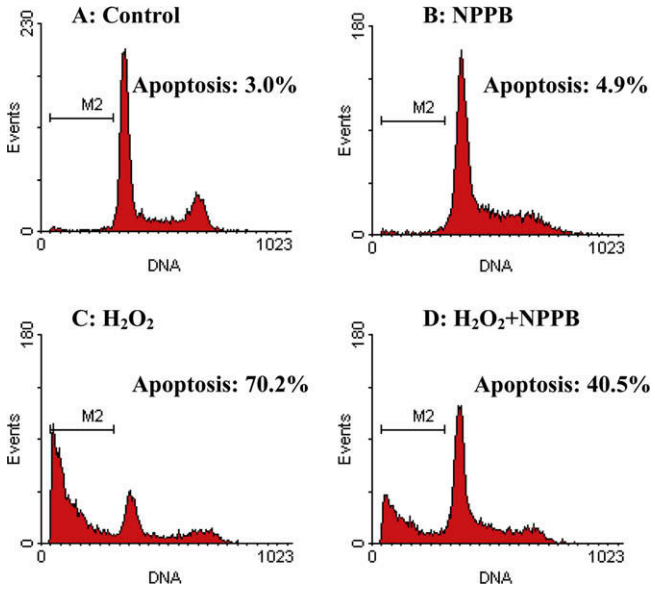

Fig. 1. The chloride channel blocker, NPPB, attenuates H_2O_2-induced apoptosis in PC12 cells-flow cytometry. Cells were incubated in the control medium (A) or treated with100 μmol/L NPPB (B), 300 μmol/L H_2O_2 (C) or 300 μmol/L H_2O_2 plus 100 μmol/L NPPB (D) for 24 h.

NPPB prevented H_2O_2-induced increment of membrane permeability to DNA dyes in early stages of apoptosis

The above results indicate that NPPB can protect cells against H_2O_2-induced apoptosis in PC12 cells. High membrane permeability is one of the characteristics of apoptotic cells. To study further, membrane permeability to the DNA dye Hoechst 33258 in the early stage of apoptosis was studied. Healthy cells emitted only weak blue fluorescence (Fig. 2A). NPPB alone did not significantly change the situation (Fig. 2B). However, most of cells (82 ± 3.6% of cells, $n = 3$) emitted strong fluorescence 1 h after exposed to 300 μmol/L H_2O_2, indicating that the membrane permeability is elevated in the early stage of H_2O_2 action (Fig. 2C). However, this elevation was prevented by the chloride channel blocker NPPB. In those treated with the mixture of 100 μmol/L NPPB and 300 μmol/L H_2O_2, only a small portion of cells emitted bright fluorescence (30.6 ± 3.0%, $n = 3$; $P < 0.01$, vs. H_2O_2 alone; Fig. 2D).

NPPB suppresses apoptotic volume decrease induced by H_2O_2

H_2O_2-induced apoptotic volume decrease in PC12 cells

As illustrated in Fig. 3A, under isotonic conditions (control), cell volume was stable ($n = 11$). When challenged with H_2O_2, cell volume decreased gradually. Cell shrinkage started as early as less than10 -min after application of H_2O_2 (300 μmol/L). At 20 and 60 min, cell volume declined to 94.8 ± 0.7% and 92.2 ± 0.9%, respectively ($n = 41$, $P < 0.01$). Cell shrinkage was worsened with the increase of H_2O_2 concentration (Fig. 3B). Application of 600 μmol/L H_2O_2 for 1 h reduced the cell volume by 19.9 ± 1.1% ($n = 28$, $P < 0.05$).

NPPB suppressed H_2O_2-induced apoptotic cell volume decrease

To further investigate the role of chloride channels in apoptotic volume decrease (AVD), we studied the effects of NPPB on H_2O_2-in-

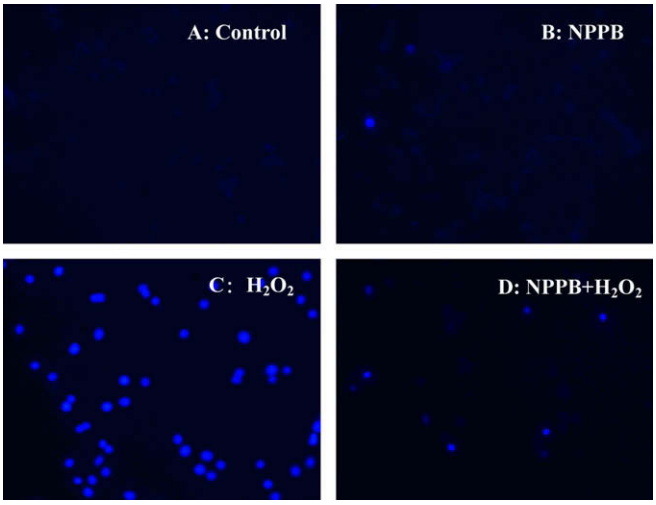

Fig. 2. NPPB attenuates H_2O_2-induced high permeability of PC12 cells to the DNA dye Hoechst 33258-florescent microscopy (200×). Fluorescent intensity of nuclei was weak in the control (A) and NPPB alone (100 μmol/L, B). Incubation with 300 μmol/L H_2O_2 for 1 h increased membrane permeability to the dye, resulting in strong fluorescence in the nuclei (C), which was prevented by 100 μmol/L NPPB (D).