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Research report

Protective effect of trifluoperazine on hydrogen peroxide-induced apoptosis in PC12 cells

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ABSTRACT

This study investigated effects of trifluoperazine (TFP) against the cytotoxicity induced by H_2O_2 in PC12 cells and the mechanisms thereof. Different concentrations of H_2O_2 (100–500 μM) induced a significant decrease in cell viability accompanied by increased oxidative stress and cell apoptosis. Pretreatment with TFP inhibited H_2O_2-induced cell viability loss. The flow cytometric assay showed that TFP can inhibit intracellular reactive oxygen species (ROS) generation and reduce the cell apoptosis. The electrophysiological recordings indicated that when treated with H_2O_2, the calcium current was significantly increased. Pretreatment with TFP increased mitochondrial membrane potential (MMP) in cells of oxidative injury. These results suggested that TFP can reduce apoptosis by inhibiting ROS generation and preventing loss of MMP in cells. Meanwhile, the protective effect of TFP on the cell apoptosis may be related to the calcium overload. TFP may inhibit the calcium overload process to achieve the protection against apoptosis.

1. Introduction

Oxidative stress-induced cell damage has long been implicated in both the physiologic process of aging and a variety of neurodegenerative diseases, such as Alzheimer's disease [16] and Parkinson's disease (PD) [17]. Prior studies indicated that oxidative stress and subsequent cell death resulted from the increased levels of hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS) through oxidative metabolism or decreased clearance of H_2O_2 due to anti-oxidant deficiency [10,15]. Compared with other organs, the brain has a high metabolic rate and relatively reduced capacity for cellular regeneration, and therefore considered particularly vulnerable to the damaging effect of H_2O_2 [7,18].

In addition, the brain has been shown to contain low to moderate levels of enzymes, such as the catalase superoxide dismutase, that plays an important role in the metabolism of ROS [4].

Calcium is one of the most important and necessary element which maintains the physiological function [27]. In neuronal tissues, Ca^{2+} signaling has been demonstrated to participate in many important activities, such as exocytosis of synaptic vesicles and neuroplasticity, but the disturbance in Ca^{2+} homeostasis has been associated with neurodegenerative processes [24]. Berridge et al. reported that the alteration of intracellular levels of Ca^{2+} can cause apoptosis [1], high levels of intracellular Ca^{2+} lead to the disruption of mitochondrial calcium equilibrium, which facilitate the loss of mitochondria membrane potential (MMP) and eventually induce the formation of ROS [23,28].

Trifluoperazine (TFP) is an inhibitor of calmodulin, which exerts more general inhibitory effects by competing for Ca^{2+} binding to specific Ca^{2+} recognition sites in proteins [6]. Kuroda et al. reported that TFP can reduce the infarct volume in transient focal brain ischemia by reducing cellular and mitochondrial Ca^{2+} overload [9]. Rodrigues et al. also reported that TFP interacted with the inner membrane of mitochondria, acquired antioxidant activity toward processes with potential implications in apoptosis, such as O_2^- accumulation, peroxidation of the membrane lipids and permeability transition process, and associated release of cytochrome *c* [20].

In the present study, the effect of TFP on H_2O_2-induced PC12 cells was investigated. The viability of the cells was observed by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays. The oxidative stress induced by H_2O_2 and their mechanism was studied in relation to the generation of ROS and intracellular Ca^{2+} concentration.

2. Materials and methods

2.1. Materials

RPMI 1640 cell culture medium was purchased from GIBCO Invitrogen. Trifluoperazine hydrochloride (TFP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 123 (Rh123), heat-inactivated horse serum, fetal bovine

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serum (FBS), H_2O_2, 4-aminopyridine (4-AP), tetraethylammonium chloride (TEA-Cl), EGTA, hepes and ATP-Na2 were all purchased from Sigma Chemical Co., St Louis, MO, USA. ROS testing kit was purchased from GENMED SCIENTIFICS, INC., USA. Annexin V-FITC propidium iodide (PI) apoptosis detection kit was from Bipec Biopharma Corporation, USA. Plastic culture microplates and flasks used in the experiment were supplied by Corning Incorporated (Costar, Corning, NY, USA). Tetrodotoxin (TTX) was obtained from the Research Institute of the Aquatic Products of Hebei (China), and other reagents were of A.R. grade.

2.2. Cell culture

PC12 rat pheochromocytoma cells were obtained from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences. The cells were maintained on plastic culture microplates with RPMI 1640 (pH 7.4) supplemented with 10% heat-inactivated horse serum and 5% FBS at 37 °C in a humidified atmosphere of 5% CO_2. All medium included 100 U/ml penicillin and 100 U/ml streptomycin.

2.3. Cell viability test

The cell viability was assessed by the methods of MTT assay described by Mosmann [13]. The cells (1×10^4/ml) were cultured with different concentrations of H_2O_2 (100–500 μM) for 12, 24 and 36 h. When the effects of TFP on PC12 cells were studied, various concentrations of TFP (0.1 μM, 1 μM and 10 μM) were added for 1 h followed by 200 μM H_2O_2 for 24 h. At the end of the exposure, 20 μl MTT was added to each well at a final concentration of 2 mg/ml and the cells were cultured for 4 h at 37 °C. The medium was then removed carefully and 150 μl DMSO was added and mixed thoroughly until formazan crystals were dissolved. The mixture was measured in an ELISA reader (Elx 800, Bio-TEK, USA) at 570 nm. The cell viability was expressed as a percentage of the control culture. Subsequently, the concentrations of TFP and H_2O_2 used in assays of ROS and apoptosis were based on the results of the MTT test.

2.4. Detection of apoptotic cells with flow cytometry

Apoptosis was assayed by annexin V-FITC and PI staining followed by analysis with flow cytometry (BECKMAN-COULTER, USA). The method followed the procedures as described in the annexin V-FITC/PI detection kit. The cells were cultured with TFP (1 μM) for 1 h before exposure to H_2O_2 in the final concentration of 200 μM for 24 h. Eventually, the cells were resuspended in 400 μl 1× binding buffer at a concentration of 1×10^6 cells/ml. The cells were then stained with 5 μl annexin V-FITC and 10 μl PI for 15 min at the room temperature in the dark. Then the cells were analyzed by flow cytometry.

2.5. Measurement of ROS

The generation of ROS for the cells was evaluated by a fluorometry assay using intracellular oxidation of DCFH-DA. The cells in logarithmic growth phase were incubated in 6-well plate for 24 h for stabilization. The different concentrations of TFP were added for 1 h prior to H_2O_2 treatment. After exposure, cells were washed with phosphate-buffered saline (PBS), then the cells were resuspended at a concentration of 1×10^6 cells/ml and were stained by the staining solution for 20 min. The cells were detected and analyzed by flow cytometry.

2.6. Measurement of mitochondrial membrane potential (MMP)

MMP was monitored using the fluorescent dye rhodamine 123 (Rh 123), a cell permeable cationic dye, which preferentially partitions into mitochondria based on the highly negative MMP [2]. Rh 123 (10 μM) was added to cell cultures for 30 min at 37 °C after cells were treated. Then the cells were collected by pipetting and washed twice with PBS, and then analyzed by flow cytometry. The mean fluorescence intensity (MFI) represents the states of depolarization of MMP in the cells.

2.7. Electrophysiological recording

2.7.1. Slice preparation and solutions

Male Wistar rats on postnatal days 10–14 were provided from Experimental Animal Center, Chinese Academy of Medical Sciences. The experiments were conducted in accordance with the guidelines of the Medical Experimental Animal Administrative Committee of Nation, and all efforts were made to minimize the number of animals used and their suffering. Horizontal slices (350 μm in thickness) which included the entire hippocampus were prepared with a vibratome (VT1000S, Leica, Germany) and incubated with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 125, $NaHCO_3$ 25, KCl 1.25, NaH_2PO_4 1.25, $MgCl_2$ 2.0, $CaCl_2$ 2.0, D-glucose 10, adjusted to pH 7.4 with NaOH. Slices were maintained in ACSF for at least 1 h before moving into the recording chamber. The slices were kept in a submerged chamber perfused with ACSF during recordings. The ACSF was saturated with 95% O_2–5% CO_2 in the experiments. All experiments were performed at room temperature (22–24 °C). The standard pipette solution for recording calcium current containing (in mM): CsCl 130, $CaCl_2$ 1, hepes 10, EGTA 10, TEA-Cl 10, Mg-ATP 3, buffered to pH 7.2 with CsOH. Tetrodotoxin (TTX, 0.001 mM), tetraethylammonium chloride (TEA-Cl, 25 mM) and 4-aminopyridine (4-AP, 3 mM) were added into the extracellular solution to block Na^+ and K^+ channels.

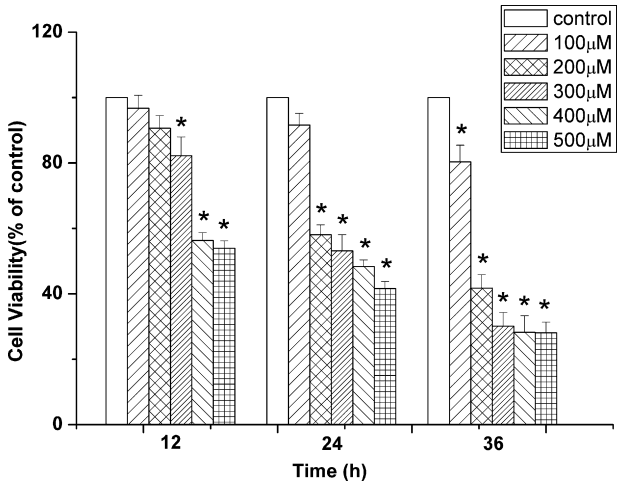

Fig. 1. Effects of different concentrations of the H_2O_2 (100, 200, 300, 400 and 500 μM) on cell viability in PC12 cells after treatment for 12, 24 and 36 h. Data were presented as mean ± S.E.M. $^*P < 0.05$ compared with untreated cells.

2.7.2. Electrophysiological recordings and drug application

For whole-cell recording, slices were transferred to a recording chamber (3 ml) placed on the stage of a modified upright infrared DIC microscope (BX51WI, Olympus, Japan). The membrane currents were measured in response to clamped voltage stimulations using a patch-champ amplifier (EPC-10, HEKA, Germany). All data were analyzed and fitted using Igor Pro 5.04 (Wavemetrics, Lake Oswego, OR, USA) and Origin 7.5 (Microcal Software, USA) software. After the establishment of a whole-cell voltage clamp configuration, the cells were allowed to stabilize for 3–5 min before starting pulse protocols to record the currents as the control. And then the final concentration of H_2O_2 (100 μM) was added into extracellular solution, respectively, once currents were stable (about 5 min) to examine the effects on the property of calcium current in rat hippocampal CA1 pyramidal neurons.

2.8. Statistical analysis

All data were expressed as mean ± S.E.M. and statistical tests were performed with one-way ANOVA followed by Dunnett's multiple comparison post hoc test. P value < 0.05 was considered significant.

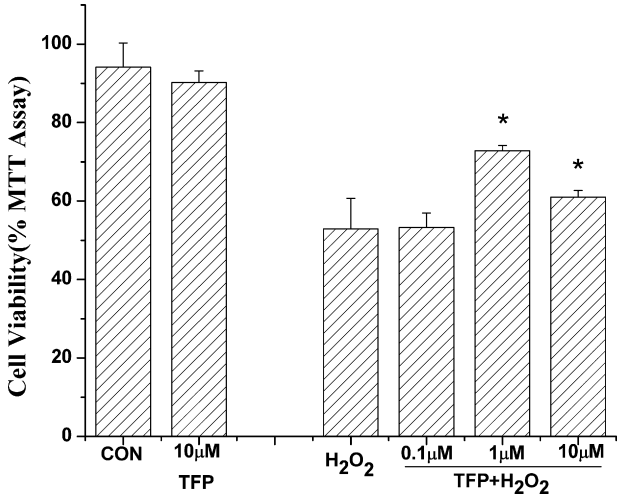

Fig. 2. Effect of TFP on H_2O_2-induced injury of PC12 cells. PC12 cells were pretreated with varying concentrations of TFP for 1 h, and 200 μM H_2O_2 was added for additional 24 h incubation. The cell viability was estimated by the MTT method. The results were expressed as percentage of the control value from the normal cells without treatments of TFP and H_2O_2. Data were presented as mean ± S.E.M. $^*P < 0.05$ compared to H_2O_2 group without TFP pretreatment.