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Cell Biology International 31 (2007) 438–443

Cell Biology International

www.elsevier.com/locate/cellbi

Neuroprotective effects of tetramethylpyrazine on hydrogen peroxide-induced apoptosis in PC12 cells

Xin-Rui Cheng [a,b], Li Zhang [a], Juan-Juan Hu [a], Lan Sun [b,*], Guan-Hua Du [a,**]

[a] *National Center for Pharmaceutical Screening, Institute of Materia Medica, Chinese Academy of Medical Science and Peking Union Medical college, 1 XianNongTan Street, Beijing 100050, PR China*

[b] *Department of Pharmacology, Institute of Basic Medical Science, Chinese Academy of Medical Science and Peking Union Medical college, Beijing 100005, PR China*

Received 29 December 2005; revised 22 September 2006; accepted 11 October 2006

Abstract

In the present study, we investigated the effects of tetramethylpyrazine (TMP) on hydrogen peroxide (H_2O_2)-induced apoptosis in PC12 cells. The apoptosis in H_2O_2-induced PC12 cells was accompanied by a decrease in Bcl-2/Bax protein ratio, release of cytochrome c to cytosol and the activation of caspase-3. TMP not only suppressed the down-regulation of Bcl-2, up-regulation of Bax and the release of mitochondrial cytochrome c to cytosol, but also attenuated caspase-3 activation and eventually protected against H_2O_2-induced apoptosis. These results indicated that TMP blocked H_2O_2-induced apoptosis by the regulation of Bcl-2 family members, suppression of cytochrome c release, and caspase cascade activation in PC12 cells.

Keywords: Tetramethylpyrazine; PC12 cells; Hydrogen peroxide; Apoptosis; Mitochondria

1. Introduction

Tetramethylpyrazine (TMP), one of the active components of traditional Chinese medicine Ligusticum Chuanxiong, is applied in the treatment of cerebralvascular and cardiovascular diseases (Lu et al., 1978; Lui, 1978). The neuroprotective effect of TMP has been demonstrated, and the possible mechanisms include inhibitory effects on calcium overload, anti-inflammatory potential. Recently studies have extended that the effect of TMP was associated with its anti-apoptotic activity. But the exact mechanism, by which TMP exerts its anti-apoptotic function, remains unclear. Clarification of the effect of TMP on PC12 cells involved in apoptosis may provide a new insight into the neuroprotection mechanism.

Numerous reports have suggested that oxidative stress plays a key role in neuronal apoptosis. It is well known that many types of chemical and physiological inducers of oxidative stress are able to cause cell apoptosis (Bayer et al., 2001; Buttke and Sandstrom, 1994). It has been demonstrated that apoptosis induced by oxidative stress is associated with the release of cytochrome c and activation of caspases (O'Brien et al., 2000; Slater et al., 1995), which plays an important role in exploring the mechanism of neuroprotective drugs. Therefore, in the present study, H_2O_2 was used as an inducer of apoptosis in PC12 cells. The changes in Bcl-2 family proteins, caspase-3 activity and the release of cytochrome c were examined, while the effects of TMP on H_2O_2-induced apoptosis were reported.

2. Materials and methods

2.1. Materials

Hoechst 33342, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and fetal bovine serum were purchased from Sigma. RPMI-1640

* Corresponding author. Tel./fax: +86 10 6529 6403.

** Corresponding author. Tel.: +86 10 6316 5184; fax: +86 10 6301 7757.

E-mail addresses: sunlan@pumc.edu.cn (L. Sun), dugh@imm.ac.cn (G.-H. Du).

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doi:10.1016/j.cellbi.2006.10.001

was obtained from Gibco. Horse serum was obtained from Hyclone. The Super-Signal West Pico Trial Kit (an enhanced chemiluminescent substrate for detection of HRP) was from PIERCE. Purified polyclonal antibodies to Bcl-2, Bax and cytochrome c were obtained from Wuhan Boster Biological Technology Co. Ltd. All other reagents were of analytical grade. TMP was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, and we confirmed in advance that TMP alone had no effect on the results of this study.

2.2. Cell culture and treatment

PC12 cells were grown on polystyrene tissue culture dishes in RPMI-1640 containing 10% horse serum and 5% fetal bovine serum, supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 95% air-5% CO_2.

To induce apoptosis, cells were incubated with the indicated concentration of H_2O_2 for different periods of time. To study the effects of TMP on PC12 cells, cells were preincubated with TMP for 4 h and H_2O_2 was added to the medium for an additional 12 h. TMP was dissolved in 50% dimethylsulfoxide (DMSO, the concentration of DMSO in the final culture medium was <0.1%, which had no effect on the cell viability).

2.3. Cell viability assay

Cell viability was measured by the method of MTT described by Mosmann (1983). Briefly, cells in 96-well plates were rinsed with phosphate-buffered saline (PBS), MTT (0.5 mg/ml) was added to each well and incubated for 4 h at 37 °C. After the medium with MTT was removed, cells and dye crystals were solubilized with 200 μl DMSO, and optical density was measured at 570 nm on a microplate reader.

2.4. Morphological assay

To observe nuclear changes occurring during apoptosis, the chromatin-specific dye Hoechst 33342 was used. Cells were harvested and fixed with 4% paraformaldyhyde for 30 min at room temperature, then washed with pre-chilled PBS three times and exposed to 10 μg/ml Hoechst 33342 at room temperature in the dark for 10 min. Samples were observed under a fluorescence microscope (Wang et al., 2005; Krohn et al., 1998).

2.5. Analysis of apoptosis rate by flow cytometry

To quantify the apoptotic cells, PC12 cells were harvested and washed twice with cold PBS and fixed with 70% ethanol. Then the cells were centrifuged at 200 × *g* for 10 min and resuspended in 100 μl PBS containing 200 μg/ml propidium iodide and 50 μg/ml RNase A at room temperature for 30 min. The fluorescence of cells was measured with a FACScan flow cytometer (Becton Dickinson, FACScan). Data were analyzed with CellQuest™ software (Ilan et al., 1994).

2.6. Caspase protease activity assay

Caspase-3-like protease activation was measured in cellular extracts with a protease assay using Ac-DEVD-AMC as a substrate (Ochu et al., 1998; Yaday et al., 1999). Briefly, after incubation, the culture media were removed, and cells were lysed in cell lysis buffer (25 mM HEPES, pH 7.5, 5 mM EDTA, 1 mM EGTA, 5 mM magnesium chloride, 10 mM sucrose, 5 mM dithiothreitol (DTT), 1% 3-[(3-cholamido-propyl) dimethylammonio]-1-propanesulfonate (CHAPS), 10 μg/ml pepstatin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were then centrifuged at 10,000 × *g* for 10 min, the supernatant was collected and its protein concentration was measured by the Lowry method. The protease assay was performed in a 96-well plate. 30 μl of protein extract was assayed in 200 μl of protease assay buffer: 20 μM Ac-DEVD-AMC, 20 mM HEPES (pH 7.5), 10% glycerol, and 2 mM DTT. Plates were read at an excitation wavelength of 498 nm and an emission wavelength of 521 nm with a FLUOSTAR plate reader (BMG Germany) after incubation at 37 °C for 2 h.

2.7. Cell fractionation and Western blot analysis

The cells were extracted in sucrose buffer supplemented with 1 mM DTT, 1 mM PMSF, and 1:1000 protease inhibitor cocktail. Lysates were homogenized and centrifuged at 500 × *g* for 12 min at 4 °C to pellet the nucleus and cell debris. The supernatant was centrifuged again at 12,000 × *g* for 20 min at 4 °C. The resulting pellet (mitochondrial fraction) was resuspended in supplemented sucrose buffer. The supernatant was further centrifuged at 15,800 × *g* for 10 min at 4 °C. The resulting pellet (cytosolic fraction) was resuspended in supplemented sucrose buffer. The protein concentration was determined by Lowry assay.

After the samples were collected, SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The separated blots were electrophoretically transferred to nitrocellulose membrane and blocked in 5% non-fat milk for 2 h at 4 °C. Blots were then incubated with primary antibody (1:500) overnight at 4 °C. The membrane was washed in PBS and incubated for 1 h at room temperature with peroxidase-conjugated goat anti-mouse antibody (1:5000). After further washing with PBS, the membrane was analyzed by the ECL method (Sano et al., 1995).

2.8. Statistical analysis

Data were expressed as the mean ± S.E.M. and evaluated by student's *t*-test. The differences were considered to be statistically significant when $P < 0.05$. Electrophoresis gel images were analyzed by the external Image Tool plug-in module ELFO described by Tomori and Molitoris and available on the Internet at http://www.saske.sk/~tomori.

3. Results

3.1. TMP prevented H_2O_2-induced viability loss in PC12 cells

It was obvious that H_2O_2 induced a time dependent viability loss in PC12 cells. Incubated with H_2O_2 at 0.12 mM for 12 h, the viability of cells was 53.2 ± 4.3% of the control value. While the viabilities of cells pretreated with TMP at 0.01 mM, 0.001 mM, 0.0001 mM were increased in a statistically significant fashion to 78.8 ± 5.2% and 73.0 ± 6.9%, 65.5 ± 4.2%, respectively (Fig. 1). The results showed that TMP effectively protected cells against H_2O_2-induced cytoxicity.

3.2. Effect of TMP on H_2O_2-induced PC12 cells apoptosis by Hoechst 33342 staining

Apoptotic cells undergo chromatin condensation, which can be visualized using the DNA-binding fluorescent dye Hoechst 33342 (Fig. 2). Nuclei of control cells appeared round to oval, with a separate pattern of blue fluorescence. After treatment by H_2O_2 at a concentration of 0.12 mM for 12 h, cell nuclei became increasingly bright. Finally it decreased in size and fragmented into apoptotic bodies. In contrast, pre-incubated with TMP, cells appeared remarkably preserved and those alterations were significantly attenuated.

3.3. TMP suppresses H_2O_2-induced apoptosis in PC12 cells

The apoptosis rate was defined as the percentage of cells with subdiploid DNA content (DNA fragmentation) determined by