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Ethyl pyruvate protects PC12 cells from dopamine-induced apoptosis

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Abstract

Pyruvate acid can protect cells against oxidative damage. However, its instability limits its usefulness as a therapeutic agent. In this study, we examined the effect of ethyl pyruvate, an aliphatic ester derived from pyruvate acid, on dopamine-induced cytotoxicity in rat pheochromocytoma PC12 cells. The results demonstrated that dopamine induced apoptosis in PC12 cells accompanied with increases of intercellular reactive oxygen species, nuclear translocation of nuclear transcription factor kappa B (NF-κB) and expression of p53 and decrease of mitochondrial transmembrane potential. Ethyl pyruvate markedly reduced the dopamine-induced production of reactive oxygen species, nuclear translocation of NF-κB, upregulation of p53, loss of mitochondrial transmembrane potential and apoptosis in PC12 cells. The results suggested that ethyl pyruvate might protect PC12 cells against dopamine by suppressing intercellular oxidative stress and modulating key signal pathways of apoptosis, and that ethyl pyruvate might be used as a potential therapeutic agent for Parkinson's disease.

Keywords: Parkinson's disease; Dopamine; Ethyl pyruvate; Oxidative stress; Nuclear transcription factor kappa B; p53

1. Introduction

Parkinson's disease is a common neurodegenerative disease, characterized by a selective loss of dopaminergic neurons of the substantia nigra. Extensive evidences suggest that oxidative stress is one of the key factors contributing to the degeneration of dopaminergic neurons (Jenner and Olanow, 1998; Marsden and Olanow, 1998; Bonnet and Houeto, 1999; Olanow and Tatton, 1999). Dopamine, an endogenous neurotransmitter, is thought to be a major source of oxidative stress in dopaminergic neuron (Olanow, 1993; Ziv et al., 1996; Offen et al., 1997). Dopamine can be oxidized by auto-oxidation spontaneously or by monoamine oxidase-mediated oxidation. Both pathways can generate free radical, hydrogen peroxide (H_2O_2), semiquinones, and quinines, which ultimately contribute to mitochondrial impairment, biomolecules oxidation and apoptosis (Cohen and Heikkila, 1974; Graham, 1978; Halliwell, 1992; Hastings, 1995; Drukarch and van Muiswinkel, 2000). Studies in vitro have shown that dopamine can induce cell death associated with the production of reactive oxygen species in variety of cell types, including rat pheochromocytoma PC12 cells (Walkinshaw and Waters, 1994), mouse neuroblastoma×rat glioma hybrid (NG108) cells (Wang and Zhu, 1995), a clonal catecholaminergic cell line (CATH.a) (Masserano et al., 1996), human neuroblastoma NMB cells (Simantov et al., 1996) and primary neuron cultures (Shinkai et al., 1997). Dopamine-induced apoptosis is protected by some antioxidants such as glutathione and *N*-acetyl-L-cysteine (Lai and Yu, 1997). However, the molecular mechanism underling dopamine-induced apoptosis has not been well established.

Pyruvate acid is a potent antioxidant and free radical scavenger (O'Donnell-Tormey et al., 1987). It is capable of scavenging H_2O_2 and OH· (Dobsak et al., 1999). Numerous studies have shown that this compound can protect cells against oxidative damage, including transient forebrain ischemia, hemorrhagic shock and reactive oxygen species-mediated acute renal failure (Salahudeen et al., 1991; Slovin

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et al., 2001; Lee et al., 2002). However, aqueous solution of pyruvate is instable and rapidly forms parapyruvate, a potent inhibitor of the mitochondrial tricarboxylic acid cycle (Montgomery and Webb, 1956; Willems et al., 1978). The instability of pyruvate largely limits its usefulness as a therapeutic agent. Ethyl pyruvate is a simple aliphatic ester derived from pyruvate acid. It was found that ethyl pyruvate was more stable and safer than pyruvate (Sims et al., 2001). Recently, a series studies have indicated that ethyl pyruvate was an effective anti-inflammatory agent in variety of in vivo and in vitro model systems (Ulloa et al., 2002; Venkataraman et al., 2002; Sappington et al., 2003). It has been shown that ethyl pyruvate attenuated lethal systemic inflammation caused by endotoxemia or sepsis in a mouse model of lethal systemic inflammation (Ulloa et al., 2002).

In the present study, the signaling pathway involved in dopamine-induced PC12 cell death was evaluated and the possible neuroprotective effect of ethyl pyruvate against dopamine-induced cell death was exploited.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and horse serum were obtained from Gibco BRL (Grand Island, NY, USA). Ethyl pyruvate, dopamine, propidium iodide, *p*-trifluoromethoxy-phenylhydrazone (FCCP), Hoechst 33258, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), *N*-acetyl-L-cysteine, catalase, trypan blue and 3-(4,5-Dimethylthiazol)-2,5-diphenyltetrazolium-bromid (MTT) were obtained from Sigma (St. Louis, MO, USA). Tetramethylrhodamine ethyl ester (TMRE) was obtained from Molecular Probes (Leiden, Netherlands). Caspase-3 substrate Ac-DEVD-AMC was obtained from Calbiochem (La Jolla, CA, USA). Monoclonal antibody against nuclear transcription factor kappa B (NF-κB) p65 subunit was obtained from Chemicon (Temecula, CA, USA). Antibodies against Bcl-2, Bax and p53 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibody against β-actine was obtained from Sigma (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit and ECL chemiluminescence system were obtained from Pierce (Rockford, IL, USA).

2.2. Cell culture and treatment

PC-12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat inactivated fetal calf serum, 10% horse serum, penicillin (25 μg/ml) and streptomycin (25 μg/ml) and were kept at 37 °C in humidified 5% CO_2/95% air. Confluent cultures were washed with phosphate-buffered saline (PBS) pH 7.4, detached with 0.5 mM EDTA, centrifuged and subcultured to poly L-lysine coated dish (cell density, 4×10^5/35-mm diameter dish and 2.5×10^6/100-mm diameter dish, respectively). Cells were seeded and grown on culture dish for 24 h before treatment. Cell viability and other morphological and biochemical markers were assessed after 1–24 h of dopamine and protective agent exposure in various experiments.

2.3. Measurement of hydrogen peroxide decomposition

Ethyl pyruvate (0.1–10 mM) was added to a reaction mixture containing: 600 μM H_2O_2, 120 mM KCl, and 50 mM Tris–HCl, pH 7.4. The reaction was performed for 30 min at 37 °C and terminated by the addition of a stopping solution (25 mg/ml of potassium biphthalate, 2.5 mg/ml of NaOH, 82.5 mg/ml of potassium iodide, and 0.25 mg/ml of ammonium molybdate). The absorbance of the mixture was measured at 350 nm. The hydrogen peroxide remaining was determined using an H_2O_2 solution as the standard (Lee et al., 2000a,b).

2.4. Cell viability assay

Briefly, the culture medium was replaced by a solution of MTT (0.5 mg/ml) in PBS supplemented with glucose (33 mM). After 3 h incubation at 37 °C, this solution was removed, and the produced blue formazan was solubilized in 1 ml of pure dimethyl sulfoxide. The optical density of the formed blue formazan was measured at 560 nm. For trypan blue staining, 0.4% trypan blue solution was added to cells collected from each dish for 3 min, and unstained live cells were counted on a hemacytometer.

2.5. Detection of fragmented DNA

DNA fragmentation was detected as described previously (Nonaka et al., 1998). In brief, the cells were lysed in 10 mM Tris–HCl (pH 7.4) containing 10 mM EDTA and 0.5% Triton X-100. The lysate was centrifuged at 12000×*g* for 12 min. Supernatant containing fragmented DNA was treated with 0.3 mg/ml proteinase K and 0.3 mg/ml RNase A at 37 °C for 1 h. DNA in the lysate was extracted with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), then with chloroform. DNA was precipitated with 2 volumes of ethanol in the presence of 0.3 M sodium acetate, followed by resuspension in 10 mM Tris–HCl (pH 8.0) containing 1 mM EDTA. The DNA was electrophoresis in 2% agrose gel in TAE buffer. The DNA bands were then visualized by ethidium bromide staining and photographed.

2.6. Nuclear changes using Hoechst 33258

To observe nuclear changes occurring during apoptosis, the chromatin-specific dye Hoechst 33258 (Sigma) was used (Krohn et al., 1999). Cultures were fixed for 10 min with 4% formaldehyde in PBS at 37 °C, and then permeabilized by treatment with a 19:1 mixture of

ethanol/acetic acid for 15 min at 20 °C. After being washed with PBS, the cells were stained with 1 μg/ml Hoechst 33258 in PBS for 20 min at room temperature and then washed again. Hoechst staining was viewed with an Axiovert 135 fluorescence microscope.

2.7. Assessment of intracellular reactive oxygen species levels

Generation of reactive oxygen species was assessed by an oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is a nonpolar compound that readily defuses into cells, where it is cleaved by intracellular esterases to form DCFH and, thereby, is trapped inside the cells. DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) by reactive oxygen species. After treatment of cells with dopamine (1 mM for 12 h), attached cells were washed with Hanks' buffer and then loaded for 60 min with 50 mg of DCFH-DA in 2 ml of culture medium. The cells were again washed three times with Hanks' buffer to remove the extracellular dye, harvested with a rubber policeman, and lysed in 0.1 M Tris containing 10% sodium dodecyl sulfate (SDS) (v/v). The lysates were sonicated for less than 10 s to reduce the viscosity of samples. The supernatants (100 μl) were assayed for DCF fluorescence with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The DCF fluorescence was then normalized based on the protein concentration of individual extracts. Protein concentration was determined with the bicinchoninic acid (BCA) assay with bovine serum albumin as the standard (Oubrahim et al., 2001).

2.8. Immunocytochemistry staining of the active p65 NF-κB subunit

Activated NF-κB was detected by the Chemicon mouse monoclonal antibody that selectively binds to an epitope overlapping the nuclear location signal of the p65 subunit of the NF-κB heterodimer. This epitope is normally inaccessible before activation because of binding of the endogenous inhibitor IκB. The specificity of the antibody for activated NF-κB has been extensively documented (Zabel et al., 1993; Kaltschmidt et al., 1995; Blondeau et al., 2001; Bui et al., 2001; Wang et al., 2002). In brief, cells grown on eight-chamber slides were fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 10 min at 4 °C. Following washes in 0.01 M PBS (pH 7.4), slides were then incubated for 1 h with 0.01 M PBS containing 1% normal horse serum, 0.1% Triton X-100 and the mouse monoclonal antibody against activated NF-κB (1:200). The immunoreaction product was then visualized according to the avidin–biotin complex method with the ABC kit. Sections were incubated with PBS containing biotinylated horse anti-mouse IgG, Triton-X and normal horse serum for 1 h and then with the PBS containing avidinbiotinylated horseradish peroxidase complex for another hour. This was followed by incubation of the slides for 4 min in diaminobenzidin solution. All steps were carried out at room temperature, and each step was followed by washes in PBS. A negative control was done using the same procedure in the absence of primary antibody. Cells were examined by light microscopy and the time course of NF-κB nuclear binding was assessed by the translocation of the p65RelA from the cytoplasm to the nucleus. The experiments described herein were repeated at least three times.

2.9. Western blot analysis

Whole cell extracts were prepared from PC12 cells by a lysis buffer (9.1 mM NaH_2PO_4, 1.7 mM Na_2HPO_4, 150 mM NaCl, pH 7.4, 1% IgepalCA-630, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail). Cell lysates were fractionated by an electrophoresis on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Each lane was loaded with an equal amount of protein extracts (20 or 60 μg), which, following electrophoresis, was transferred to a polyvinylidene difluoride membrane for 1.5 h. Blots were stained with Ponceau S to verify equal loading and transfer of proteins. Nonspecific binding was blocked by soaking the membrane in TBST buffer (20 mM Tris base, pH 7.5, 137 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk overnight. Membranes were then probed with anti-Bcl-2, anti-Bax, anti-p53 antibody (Santa Cruz Biotechnology) (1:1000) for 3 h. After washing with TBST, the membrane was then incubated with goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (1:10,000) for 1 h. Intensity of the signal was determined by ECL chemiluminescence system (Pierce Biotechnology, Rockford, IL) and Kodak X-ray film (Konica, Tokyo, Japan). Incubation with monoclonal anti-β-actin (1:5000 dilution; Sigma), was performed for comparative control.

2.10. Reverse transcriptase–polymerase chain reaction

Total RNA was extracted by Trizol-Reagent (Life Technologies), according to manufacturer's instructions. Aliquots of total RNA (2 mg) were reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (M-MLV) (Promega, Madison, WI) and oligo (dT) primer (18-mer). Aliquots of the obtained cDNAs were then amplified by PCR performed in 15 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTPs, 2.5 U DNA polymerase, and 10 pmol of each primer. The reaction conditions were as follows: warm up period of 5 min at 95 °C, cycles of PCR (95 °C for 45 s; 58 °C for 45 s for Bax, Bcl-2 and p53 or 63 °C for 45 s for induced nitric oxide synthase (iNOS) and β-actin; 72 °C for 1 min) and a final elongation period of 10 min at 72 °C. The cycles were performed 24 times for Bax and p53, 21 times for β-actin and 35 times for iNOS, respectively. All primers were pre-

tested with an increasing number of amplification cycles for obtaining reverse transcriptase–polymerase chain reaction (RT–PCR) products in the exponential range under the above conditions.

Sequences of primer:

p53: Forward: 5′-GCC ATC TAC AAG AAG TCA CA-3′; Reverse: 5′-GTC TTC CAG CGT GAT GAT G-3′;
Bcl-2: Forward: 5′-CCG,GGA,GAT,CGT,GAT,-GAA,GTA-3′; Reverse: 5′-CAT,ATT,TGT,TTG,GGG, CAT,GTCT-3′;
Bax: Forward: 5′-GCA,GGG,AGG,ATG,GCT,GGG, GAG,A-3′; Reverse: 5′-TCC,AGA,CAA,GCA,GCC, GCT,CAC,G-3′;
iNOS: Forward: 5′-CTGCATGGAACAGTATAAGG-CAAAC-3′; Reverse: 5′-CAGACAGTTTCTGGTC-GATGTCATGA-3′;
β-Actin: Forward: 5′-AAGATGACCCAGATCATGTT-3′; Reverse: 5′-TTAATGTCACGCACGATT-3′.

2.11. Determination of mitochondrial membrane potential

To evaluate mitochondrial membrane potential (Δψm), exponentially growing cells on cover slips were incubated with 1 mM dopamine in absence or presence of 5 mM ethyl pyruvate for 12 h at 37 °C. At the end of incubation, the medium was removed, and attached cells were labeled with TMRE (a dye that accumulates in mitochondria in response to Δψm) at a concentration of 100 ng/ml of culture medium for 15 min at room temperature. After washing, cells were immediately analyzed with a fluorescence microscope. Alternatively, attached cells were scraped and lysed in a buffer of 0.1 M Tris containing 10% SDS (v/v), and the TMRE fluorescence was measured with a microplate reader as described above. The excitation and emission wavelengths were set at 508±20 nm and 580±40 nm, respectively. The fluorescence was normalized for the protein concentration of individual extracts. To ensure that mitochondrial uptake of TMRE was related to membrane potential, a control study was carried out simultaneously with an uncoupling agent (FCCP 10 μM) that is known to abolish the m. Protein concentration was determined with the BCA assay with bovine serum albumin as the standard (Oubrahim et al., 2001). All experiments described herein were repeated at least three times.

2.12. Caspase-3-like protease activation

Caspase-3-like protease activation was measured in cellular extracts with a protease assay using Ac-DEVD-AMC as a substrate (Krohn et al., 1999). Briefly, 2×10^6 cells were lysed in cell lysis buffer (10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 5 mM DTT, 1 mM PMSF, 10 μg/ml pepstatin A, 20 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell extracts were then centrifuged at 14,000 rpm for 10 min, supernatants were transferred to new tubes, and protein concentration was measured by the BCA assay. The protease assay was performed in a 96-well plate. Thirty microliters 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 incubated at 37 °C for 0.5, 1, 1.5, 2 and 2.5 h and then read at 460 nm with a FLUOstar plate reader (BMG Germany).

2.13. Statistics

Data are presented as means±S.E.M. of three independent experiments. Statistical analysis was performed by applying the Student's *t*-test. *P* values of <0.01 were considered to be significant.

3. Result

3.1. Effect of dopamine on PC12 cell viability

After PC12 cells were treated with dopamine (0.1–1 mM) for 24 h, a concentration-dependent decrease of cell viability was found by MTT reduction assay (Fig. 1A) and trypan blue dye exclusion assay (Fig. 1B). Dopamine-induced PC12 cell death had the typical feature of apoptosis, as judged by morphological nuclear changes and DNA fragmentation. Characteristic ladder of oligonucleosomal DNA fragment and condensed cell nucleic were evidently shown when PC12 cells were treated with 1 mM dopamine for 18 h (Figs. 2A and 5). Thus, 1 mM dopamine was used in the following studies.

3.2. Effect of ethyl pyruvate on dopamine-induced neurotoxicity

After PC12 cells were co-treated with dopamine (1 mM) and ethyl pyruvate (0.1–10 mM) for 24 h, ethyl pyruvate produced a concentration-dependent inhibition of cell death (Fig. 3). Incubation with ethyl pyruvate at 1 mM and 5 mM increased cell viability up to 50% and 90%, respectively. The maximal neuroprotection was achieved by 5 mM ethyl pyruvate. *N*-acetyl-L-cysteine (10 mM) and catalase (100 U/ml) also significantly protected dopamine-induced cell death (Fig. 3). When PC12 cells were treated with dopamine, the incubation medium turned its color to orange-brown. In the presence of *N*-acetyl-L-cysteine, the dopamine-induced color change was abolished. However, co-treatment with ethyl pyruvate or catalase had no effect on the color change induced by dopamine. The results suggested that auto-oxidation of dopamine might be involved when cells were treated with dopamine and that *N*-acetyl-L-cysteine but not ethyl pyruvate or catalase protected the spontaneous auto-oxidation of dopamine. Morphological analysis under light microscopy showed that dopamine induced severe cell

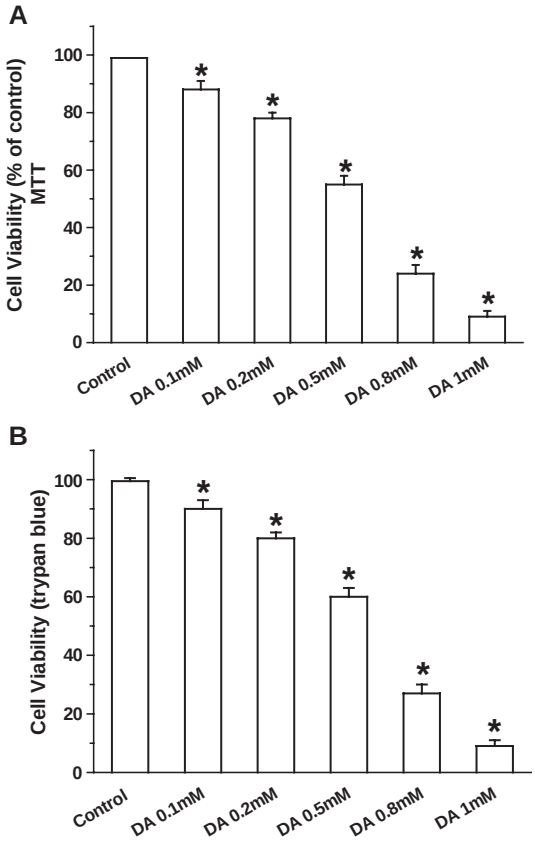

Fig. 1. Effect of dopamine on PC12 cell viability. (A) PC12 cells were treated with dopamine (DA, 0.1–1 mM) for 24 h. Cell viability was determined by MTT colorimetric assay. Results are expressed as the percentage of surviving cells compared with control cultures. (B) PC12 cells were treated with dopamine (DA, 0.1–1 mM) for 24 h. Cell viability was determined by trypan blue dye exclusion assay. Results are expressed as the percentage of surviving cells relative to total cells. Data are mean±S.E.M. of three independent experiments. *P<0.01 compared with control cultures.

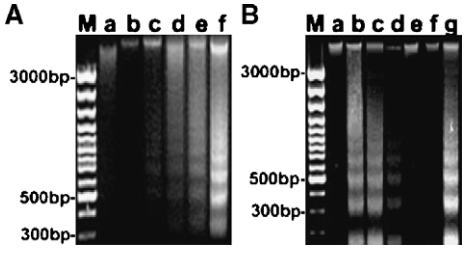

Fig. 2. Effect of ethyl pyruvate on dopamine-induced DNA fragmentation in PC12 cells. (A) PC12 cells were treated with dopamine (0.1–1 mM) for 18 h. Gel lane: M: maker; a: control; b: dopamine (0.1 mM); c: dopamine (0.2 mM); d: dopamine (0.5 mM); e: dopamine (0.8 mM); f: dopamine (1 mM). (B) PC12 cells were treated with dopamine (1 mM) in the presence of ethyl pyruvate (0.1–10 mM) for 18 h. Ethyl pyruvate produced a concentration-dependent inhibition of apoptosis. Gel lane: M: maker; a: control; b: dopamine (1 mM)+ethyl pyruvate (0.1 mM); c: dopamine (1 mM)+ethyl pyruvate (0.5 mM); d: dopamine (1 mM)+ethyl pyruvate (1 mM); e: dopamine (1 mM)+ethyl pyruvate (5 mM); f: dopamine (1 mM)+ethyl pyruvate (10 mM). g: dopamine (1 mM).

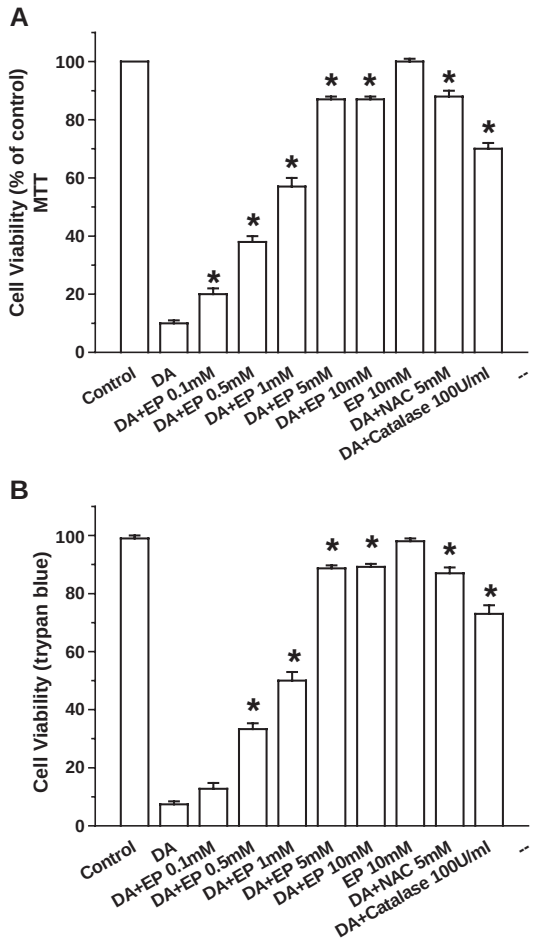

Fig. 3. Effect of ethyl pyruvate on dopamine-induced cell death in PC12 cells. PC12 were treated with dopamine (DA, 1 mM) and ethyl pyruvate (EP, 0.1–10 mM) or *N*-acetyl-L-cysteine (NAC, 5 mM) or catalase (100 U/ml) for 24 h. Data are mean±S.E.M. of three independent experiments. *P<0.01 compared with PC12 cells treated with dopamine alone. (A) MTT assay; (B) Trypan blue dye exclusion assay.

damage as evidenced by a decrease in the cell size and loss the bright refringent halo around cell bodies (Fig. 4). Ethyl pyruvate protected dopamine-induced morphological changes in a concentration-dependent manner (Fig. 4). However, ethyl pyruvate itself had no effect on the cell viability of PC12 cells. Co-treatment with ethyl pyruvate (0.1–10 mM) for 18 h also attenuated dopamine-induced apoptosis in concentration-dependent manner as evidenced by a significant reduction of internucleosomal DNA fragmentation (Fig. 2B) and apoptosis nuclei (Fig. 5).

3.3. Effect of ethyl pyruvate on hydrogen peroxide degradation

To examine the defensive effect of ethyl pyruvate against oxidative attack, the decomposing effects of ethyl pyruvate on H_2O_2 were observed. H_2O_2 is attained from the dismutation of the superoxide anion and is well known as a precursor of highly reactive oxidant. As shown in Fig. 6,

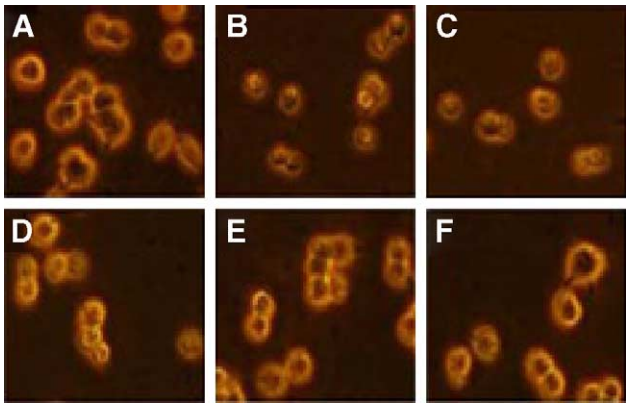

Fig. 4. Effect of ethyl pyruvate on dopamine-induced morphological change in PC12 cells. PC12 cells were treated with dopamine (1 mM) in the presence of ethyl pyruvate (0.1–5 mM) for 24 h. A: Control; B: dopamine (1 mM); C: dopamine (1 mM)+ethyl pyruvate (0.1 mM); D: dopamine (1 mM)+ethyl pyruvate (0.5 mM); E: dopamine (1 mM)+ethyl pyruvate (1 mM); F: dopamine (1 mM)+ethyl pyruvate (5 mM).

ethyl pyruvate directly decomposed hydrogen peroxide in a concentration-dependent manner.

3.4. Effect of ethyl pyruvate on dopamine-induced reactive oxygen species production

When PC12 cells were treated with 1 mM dopamine for 12 h, a 1.9 fold increase in the intercellular reactive oxygen species was found using DCFH-DA assay. Co-incubation with dopamine and ethyl pyruvate decreased the reactive oxygen species production (Fig. 7). Ethyl pyruvate at concentration of 10 mM completely prevented dopamine-induced reactive oxygen species production. The results suggest that reactive oxygen species is generated during dopamine-induced PC12 cells apoptosis and that ethyl pyruvate may act as a potential candidate to attenuate dopamine-induced reactive oxygen species production.

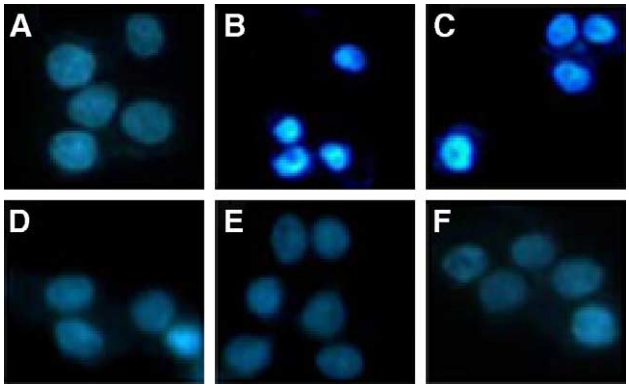

Fig. 5. Effect of ethyl pyruvate on dopamine-induced apoptosis assessed by Hoechst 33258 staining in PC12 cells. PC12 cells were treated with dopamine (1 mM) in the presence of ethyl pyruvate (0.1–10 mM) for 18 h. Apoptosis cell death was assessed by Hoechst 33258 staining. A: Control; B: dopamine (1 mM); C: dopamine (1 mM)+ethyl pyruvate (0.5 mM); D: dopamine (1 mM)+ethyl pyruvate (1 mM); E: dopamine (1 mM)+ethyl pyruvate (5 mM); F: dopamine (1 mM)+ethyl pyruvate (10 mM).

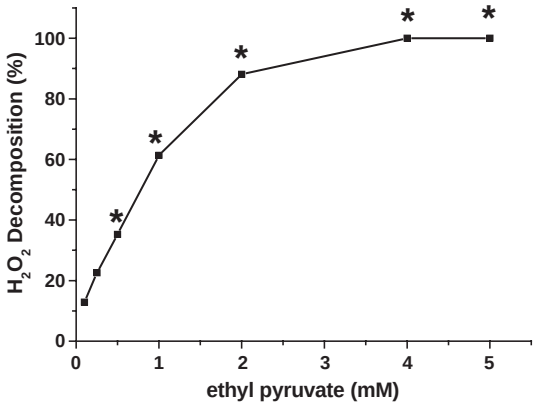

Fig. 6. Decomposition effect of ethyl pyruvate on H_2O_2. Ethyl pyruvate (0.1–10 mM) was added to a reaction mixture containing: 600 μM H_2O_2, 120 mM KCl, and 50 mM Tris–HCl, pH 7.4. The reaction was performed for 30 min at 37 °C and terminated by the addition of a stopping solution (25 mg/ml of potassium biphthalate, 2.5 mg/ml of NaOH, 82.5 mg/ml of potassium iodide, and 0.25 mg/ml of ammonium molybdate). The absorbance of the mixture was measured at 350 nm. The hydrogen peroxide remaining was determined using an H_2O_2 solution as the standard. *P<0.01 vs. control.

3.5. Effect of ethyl pyruvate on dopamine-induced NF-κB nuclear translocation

The time course of nuclear translocation of activated NF-κB was examined after 1–12 h exposures of PC12 cells to 1 mM dopamine. There was a significant overall effect of dopamine treatment over time on the nuclear translocation of activated NF-κB. Nuclear NF-κB were detected in some cells after 1 h dopamine exposure and increased at each successive time point up to 12 h. After 12 h exposure, NF-κB was detected in approximately 78% of cell nuclei (Fig. 8). However, in cells co-incubated with

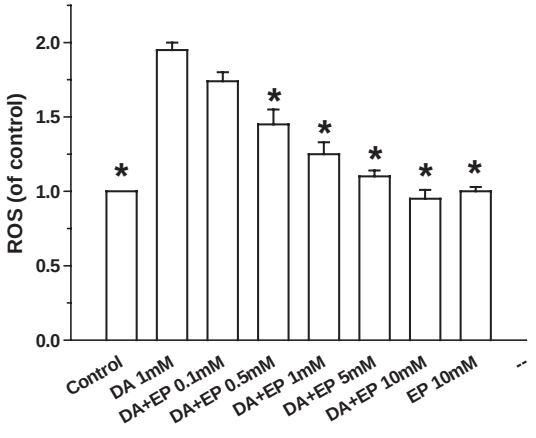

Fig. 7. Effect of ethyl pyruvate on dopamine-induced reactive oxygen species production in PC12 cells. PC12 cells were treated for 12 h with dopamine (DA, 1 mM) in the presence of ethyl pyruvate (EP, 0–10 mM) and then stained with DCFH-DA (10 μM) for 30 min. The DCF fluorescence was measured by fluorometry. Results are expressed as the percentage of reactive oxygen species (ROS) production in each group compared with control. Data are mean±S.E.M. of three independent experiments. *P<0.01 compared with group treated with dopamine alone.

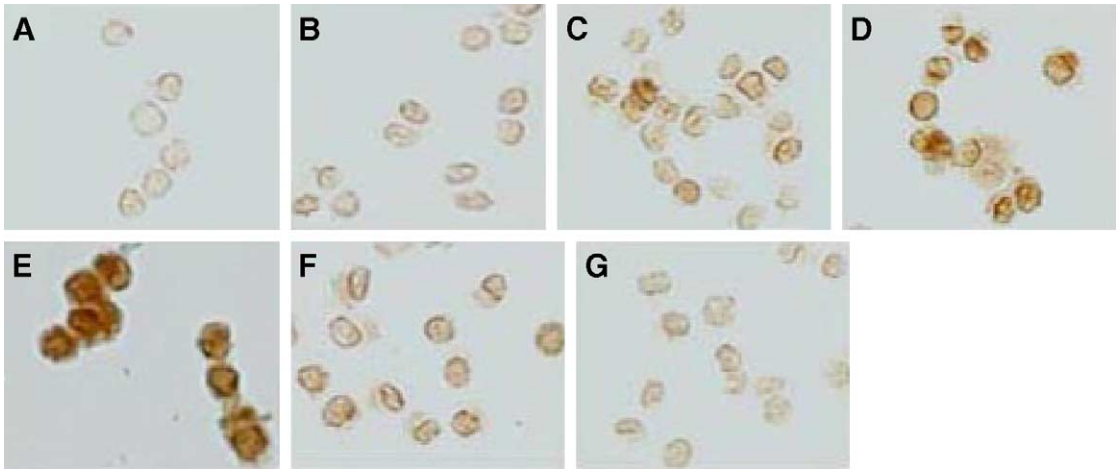

Fig. 8. Effect of ethyl pyruvate on dopamine-induced NF-κB activation and nuclear translocation in PC12 cells. The time course of nuclear translocation of activated NF-κB was examined after 1–12 h exposures of PC12 cells to 1 mM dopamine. A: Negative control with omission of first antibody; B: control; C: dopamine (1 mM) for 1 h; D: dopamine (1 mM) for 3 h; E: dopamine (1 mM) for 12 h; F: dopamine (1 mM)+ethyl pyruvate (5 mM) for 12 h; G: ethy pyruvate (5 mM) for 12 h.

ethyl pyruvate (5 mM) and dopamine for 12 h, activated NF-κB was rarely detected in nuclei. Ethyl pyruvate treatment markedly attenuated the translocation of NF-κB to the nucleus (Fig. 8).

To investigate the modulation of activated NF-κB on the expression of its downstream genes, the expression of iNOS known to be regulated by NF-κB was examined using RT–PCR. Low level transcription of iNOS was observed after 4 h of dopamine incubation and then lasted at least 6 h (Fig. 9). However, addition of ethyl pyruvate (5 mM) completely inhibited the transcription of iNOS induced by dopamine (Fig. 9).

3.6. Effect of ethyl pyruvate on dopamine-induced p53 and Bax upregulation

It has been well known that the nuclear transcription factor p53 is regulated in response to cellular stress, including oxidative stress (Morrison and Kinoshita, 2000). Therefore, the cellular levels of p53 mRNA and protein were measured after dopamine treatment, using RT–PCR and Western blot assays. Both transcription and expression of p53 were observed in untreated control. After PC12 cells were exposed to dopamine, the p53 mRNA and protein levels were augmented at 4 h and then maintained at high level for at least 8 h (Fig. 10A and C). Addition of 5 mM ethyl pyruvate attenuated the upregulation of p53 induced by dopamine (Fig. 10B and D).

Bax has been implicated in promoting apoptosis, and its transcription is upregulated by p53 (Miyashita and Reed, 1995). In this study, the transcription and expression of Bax were further measured in response to dopamine and ethyl pyruvate treatment. The level of Bax was significantly increased in PC12 cells treated with dopamine for 6 h, following the increase of p53 (Fig. 10A and C). The upregulation of Bax was still observed at 12 h after treatment of dopamine. Ethyl pyruvate (5 mM) attenuated the upregulation of Bax (Fig. 10B and D). Ethyl pyruvate (5 mM) alone had no effect on the expression of p53 and Bax as shown in Fig. 10B and D. In the present studies, no Bcl-2 transcription or expression was detected in untreated or ethyl pyruvate and/or dopamine-treated PC12 cells.

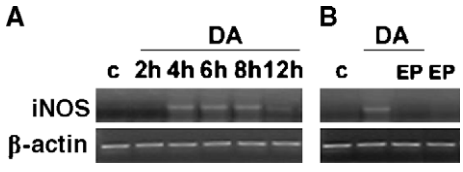

Fig. 9. Effect of ethyl pyruvate on dopamine-induced iNOS mRNA expression in PC12 cells. (A) The time course of iNOS mRNA expression induced by dopamine (DA, 1 mM) in PC12. (B) Effect of ethyl pyruvate (EP, 5 mM) on iNOS mRNA expression in PC12 cells treated with dopamine (DA, 1 mM) for 8 h. Results were representative of three independent experiments.

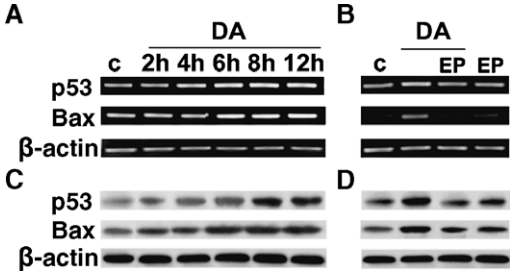

Fig. 10. Effect of ethyl pyruvate on dopamine-induced p53 and Bax expression in PC12 cells. (A) Time course of p53 and Bax mRNA expression induced by dopamine (DA, 1 mM). (B) Effect of ethyl pyruvate (EP, 5 mM) on p53 and Bax mRNA expression in PC12 cells treated with dopamine (DA, 1 mM) for 8 h. (C) Time course of p53 and Bax protein expression in PC12 cells induced by dopamine (DA, 1 mM). (D) Effect of ethyl pyruvate (EP, 5 mM) on p53 and Bax protein expression in PC12 cells treated with dopamine (DA, 1 mM). The p53 and Bax mRNA expression were measured by RT–PCR and p53 and Bax protein expression were measured by western blot analyses as described in Materials and methods.

3.7. Effect of ethyl pyruvate on dopamine-induced Δψm alterations and caspase-3 activation

It is implied that Bax can induce loss in mitochondrial transmembrane potential (Δψm) and cytochrome *c* release, which promotes cell apoptosis (Tsujimoto, 1998; Shimizu et al., 1999). Release of cytochrome *c* to cytosol is involved in the activation of apoptosis executers, caspase-3 and caspase-9. Therefore, the Δψm alterations and caspase-3 activity in treated and untreated PC12 cells were measured in the present studies. Binding of the fluorence dye, TMRE, in a variety of mammalian cells has been shown to depend on high transmembrane potential maintained in functional mitochondria. In comparison with controls, there was no decline in TMRE fluorescence, indicative of mitochondrial depolarization, up to 8 h after the onset of dopamine exposure (data not shown). On the contrary, dopamine-treated cells showed a significant decrease in TMRE fluorescence at 12 h, and this fluorescence decrease in cells could be partially restored by the addition of ethyl pyruvate (5 mM) (Fig. 11). In an effort to determine whether this difference in TMRE uptake reflected a difference in mitochondrial transmembrane potential, FCCP, a compound known to disrupt transmembrane potential of mitochondria was used to monitor the TMRE-mitochondria interaction in untreated cells. Indeed, addition of FCCP was accompanied by a huge decrease in TMRE fluorescence (Fig. 11). These results suggest that ethyl pyruvate protected PC12 cells against dopamine-induced Δψm loss.

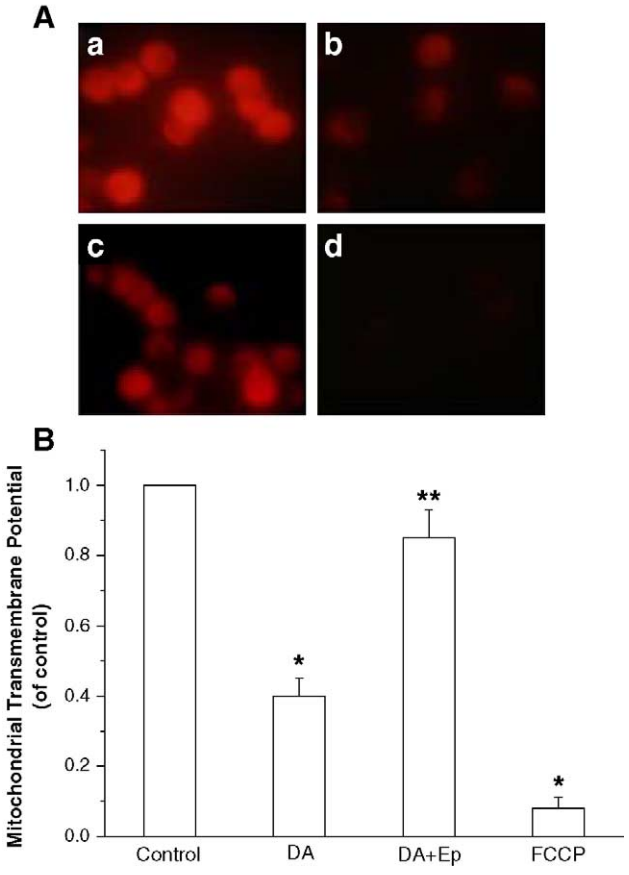

Fig. 11. Effect of ethyl pyruvate on dopamine-induced m alterations in PC12 cells. PC12 cells were treated with dopamine (DA, 1 mM) in the presence or absence of ethyl pyruvate (EP, 5 mM) for 12 h. After treatment, cells were loaded with TMRE and then analyze with a fluorescence microscope. (A) Representative fluorescence microscope pictures. a: Control; b: dopamine (1 mM); c: dopamine (1 mM)+ethyl pyruvate (5 mM); d: FCCP (10 μM). (B) TMRE fluorescence density measured by a fluorometer. PC12 cells were treated with dopamine (DA, 1 mM) or dopamine (1 mM)+ethyl pyruvate (5 mM) (DA+EP) or with FCCP. Data are expressed after normalizing for protein content, untreated control was considered as 1. All data are expressed as the means of three independent experiments. *P<0.01 compared with untreated control, **P<0.01 compared to group treated with dopamine alone.

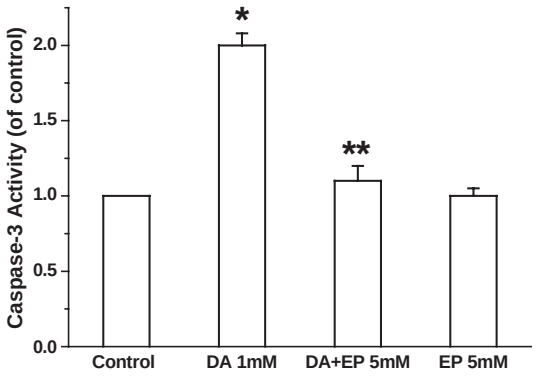

Fig. 12. Effect of ethyl pyruvate on dopamine-induced activation of caspase-3-like activity in PC12 cells. PC12 cells were treated for 12 h with dopamine (DA, 1 mM) alone or in combination with ethyl pyruvate (EP, 5 mM). Data are mean±S.E.M. from three independent experiments. *P<0.01 compared with control; **P<0.01 compared with dopamine treated group.

When caspase-3 activity was measured fluorometrically by monitoring cleave of the caspase-3 substrate Ac-DEVD-AMC in the cytosolic fraction of cells, it was found that caspase-3 activity was not altered during the first 8 h of dopamine treatment (data not show), however, caspase-3 activity was elevated up to 2-fold of the control values after 12 h dopamine exposure. The dopamine-induced increase of caspase-3 activity was significantly inhibited in the presence of ethyl pyruvate (5 mM) (Fig. 12). Ethyl pyruvate alone had no effect on the caspase-3 activity.

4. Discussion

In the present studies, dopamine was used to induce PC12 cell death as a model of substantia nigral degeneration in Parkinson's disease. We demonstrated that dopamine induced a concentration-dependent cell death within a period of 24 h. In agreement with previous studies (Offen et al., 1997; Panet et al., 2001; Chen et al., 2003), our studies demonstrated that dopamine induced PC12 cells death with apoptotic characteristics, as judged by morphological nuclear changes, DNA fragmentation and caspase-3

activation. The apoptotic death of dopaminergic cells was associated with an increase of intercellular reactive oxygen species followed by activation of NF-κB, upregulation of p53 and Bax, loss of mitochondrial transmembrane potential and caspase-3 activation. The dopamine-induced PC12 cell death was prevented by ethyl pyruvate treatment. The neuroprotection of ethyl pyruvate against dopamine-triggered apoptosis might be related to its capability of scavenging reactive oxygen species and subsequent inhibition of apoptosis signal pathway.

Oxidative stress is thought to be involved in the death of neurons in the substantia nigra of Parkinson's disease (Ziv et al., 1996; Bonnet and Houeto, 1999). The finding that the oxidative metabolism of dopamine generates reactive oxygen species suggested that dopamine might be the major source of oxidative stress in dopaminergic neuron (Ziv et al., 1996; Wang and Zhu, 2003; Liu et al., 2005). The present study demonstrated that dopamine-induced apoptosis in PC12 cells was accompanied by an increase of reactive oxygen species production and reversed by *N*-acetyl-L-cysteine and catalase. These results favor the view that oxidative damage is mainly responsible for the cytotoxicity of dopamine. The finding that catalase, an enzyme which promotes the conversion of H_2O_2 to H_2O and O_2, markedly attenuated dopamine-induced toxicity strongly suggests that H_2O_2 might be the major substance involving in dopamine-induced oxidative damage. In the present studies, ethyl pyruvate was found to induce a concentration-dependent protection against dopamine-induced cell death. The protection of ethyl pyruvate against dopamine-induced apoptosis might be related to its radical-scavenging capacity. This view was confirmed by the finding that ethyl pyruvate could directly decompose hydrogen peroxide and attenuate dopamine-induced intercellular reactive oxygen species production. In the present studies, we found that the color of the incubation medium was changed when PC12 cells were treated with dopamine, indicating that auto-oxidation of dopamine might be involved. The auto-oxidation of dopamine was attenuated by *N*-acetyl-L-cysteine, an antioxidant. However, neither ethyl pyruvate nor catalase had effect on the dopamine-induced color change, suggesting that ethyl pyruvate and catalase had no suppressive effect on the dopamine auto-oxidation. Evidence has shown that hydrogen peroxide can react with trace metals to form highly reactive hydroxyl radical ($OH\cdot$) (Fenton, 1894) and then oxidatively damage proteins, lipids and DNA (Halliwell, 1992). Evidence has also demonstrated that ethyl pyruvate was capable of scavenging hydroxyl radical ($OH\cdot$) (Varma et al., 1998). However, hydroxyl free radical scavengers failed to affect dopamine toxicity (Lai and Yu, 1997). Taken together, ethyl pyruvate might protect PC12 cells against dopamine toxicity mainly by scavenging H_2O_2. In contrast to catalase, ethyl pyruvate can penetrate cell membranes, thus scavenge both extracellular and intercellular H_2O_2 and therefore protect cells from damage.

NF-κB, one of the transcription factors known to be activated by oxidative stress, has gained increasing attention for its role as a regulator of cell death (Barkett and Gilmore, 1999). NF-κB activity can be either pro- or anti-apoptotic, depending upon the experimental conditions and target proteins involved (Qin et al., 1998; Yu et al., 2000; Panet et al., 2001; Yabe et al., 2001; Wang et al., 2002). NF-κB activation has been implicated in Parkinson's disease neurodegeneration by Hunot et al. (1997), who found an enhanced nuclear translocation of NF-κB in post-mortem dopaminergic brain tissue of patients with Parkinson's disease. Others toxins such as 6-hydroxydopamine, MPP^+ and rotenone which selectively insulted dopaminergic neuron, also induced NF-κB activation (Cassarino et al., 2000; Blum et al., 2001; Wang et al., 2002). However, the functional significance of NF-κB activation in Parkinson's disease remains unclear. Recently, there is a line of evidence indicating that NF-κB is not only activated in dopamine-induced apoptosis of PC12 cells but also mediated the apoptotic process (Panet et al., 2001). In agreement with these finding, we demonstrated that NF-κB activation and nuclear translocation were involved in dopamine-induced PC12 apoptosis. Furthermore, we found that the transcription of iNOS, the downstream of NF-κB activation, was induced during the apoptosis. The nuclear localization of activated NF-κB was occurred as early as 1 h after dopamine exposure and continued to increase over 12 h, while the expression of iNOS was observed at 4 h after dopamine treatment. The relative time course of these two events indicated that NF-κB nuclear translocation preceded the expression of iNOS. The finding that NF-κB activation and iNOS expression were attenuated in the presence of ethyl pyruvate strongly suggested that dopamine-induced iNOS expression was regulated by activated NF-κB.

NO is an important mediator of MPTP toxicity in dopaminergic neurons (Dehmer et al., 2004). NO also involved in dopamine-induced PC12 cells apoptosis (Chen et al., 2003). We demonstrated that incubation of PC12 cells with dopamine led an increase in iNOS transcription, whereas pretreatment with aminoguanidine, an iNOS inhibitor, almost completely inhibited iNOS protein expression and NO generation and subsequent cell apoptosis (Chen et al., 2003). The finding that NF-κB induced expression of a toxic protein, iNOS and ethyl pyruvate inhibited both dopamine-induced nuclear binding of NF-κB and apoptosis gives a direct evidence to support the hypothesis that NF-κB activation is involved in dopamine-induced apoptosis.

The preventive effect of ethyl pyruvate on dopamine-induced NF-κB activation might be attributed to its antioxidative capability. However, the other pathways mediating ethyl pyruvate-induced NF-κB inhibition cannot be excluded. It has been known that nuclear translocation of the transcription factor NF-κB is a critical component of the cellular stress response to multiple stimuli including irradiation, cytokines, chemical toxin and oxidative stress.

Weingarten et al. (2001) found that dopamine could stimulate NF-κB translocation by several mechanisms including a non-oxidative activation of NF-κB. Furthermore, agents that could modify free sulfhydryls such as *N*-ethylmaleimide and diamide inactivated the DNA binding of NF-κB (Brennan and O'Neill, 1998). In LPS-stimulated RAW 264.7 cells, the inhibition of LPS-induced NF-κB DNA binding by ethyl pyruvate was proved to be related to the depletion of GSH. Addition of GSH partially reversed the effects of ethyl pyruvate on the inhibition of NF-kB DNA binding (Song et al., 2004). However, further study will be needed to elucidate the precise mechanism by which ethyl pyruvate inhibited the dopamine-induced NF-κB activation.

Evidence has shown that p53 induced cell death by a multitude of molecular pathways involving transactivation of target genes and direct signaling events (Li et al., 1999; Lee et al., 2000a,b). Genes transcriptionally upregulated by p53 that have been implicated in promoting apoptosis include Bax, Bak, and Noxa. It has been found that Bax function was required for the release of cytochrome *c* from the mitochondria to the cytosol during apoptosis (Tsujimoto, 1998) and that the release of cytochrome *c* subsequently activated caspase-3 and -9. Previous studies have demonstrated that caspase-3 inhibitor could rescue PC12 cells from dopamine damage (Panet et al., 2001). In the present studies, we demonstrated that upregulation of p53 in mRNA and protein levels was first occurred at 4 h after dopamine exposure, while the upregulation of Bax was detected at 6 h after dopamine exposure. Alteration of Bax expression delayed 2 hours compared to p53. However, the upregulation of p53 and Bax preceded the loss of mitochondrial transmembrane potential and the increase of caspase-3 activity. Ethyl pyruvate inhibited the dopamine-induced upregulation of p53 and subsequent activation of apoptosis cascades. Thus, our results indicate that p53 might mediate dopamine-induced PC12 cells apoptosis. The upregulation of p53 might be triggered by reactive oxygen species generated by dopamine metabolism.

Mitochondria-mediated apoptosis has been shown to be associated with the reduction in $\Delta\psi$m (Zamzami et al., 1996). In the present study, we demonstrated that dopamine treatment caused a marked loss in m and that the loss in $\Delta\psi$m could be restored by ethyl pyruvate in PC12 cells. The result indicated that mitochondria were target involved in dopamine-induced PC12 cell apoptosis. Oxidized metabolites of dopamine have been shown to inhibit the mitochondrial respiratory system both in vivo and in vitro (Berman and Hastings, 1999). Recently, Ben-Shachar et al. (2004) reported that dopamine toxicity involved, or was initiated by, its interaction with the mitochondrial oxidative phosphorylation system. Since pyruvate is a key intermediate in the oxidative or anaerobic metabolism, ethyl pyruvate, as a pro-drug of pyruvate, might directly protect mitochondria from dopamine damage by ameliorating impairment of ATP production, the main function of mitochondria.

Treatment with pyruvate has been proved to be salutary in cardiac function, coronary heart disease and ischemic/reperfusion injury (Bunger et al., 1989; Lee et al., 2002). Unfortunately, pyruvate acid is unstable in solution, which limits its usefulness as a therapeutic agent in clinical treatment. Ethyl pyruvate, a simple derivative of pyruvate, is found to be safe, stable and easy to penetrate tissue membrane. Research regarding its therapeutic application has primarily focused on its anti-inflammation effects. These studies have clearly indicated that ethyl pyruvate could effectively protect several tissues against various inflammation injuries (Ulloa et al., 2002; Venkataraman et al., 2002; Sappington et al., 2003). Since ethyl pyruvate can simultaneous act as energy substrate, a potent free radical scavenger and a powerful anti-inflammatory agent, ethyl pyruvate might have therapeutic application in neurological injury. With the characteristic pathology of Parkinson's disease involving synchronized oxidative stress, mitochondrial aberration and inflammation in the substantia nigra region of the brain (Beal, 2003), we suggest that ethyl pyruvate might be a potential therapeutic agent for deterring neurodegeneration in Parkinson's disease. To evaluate this possibility, further studies in animal model of Parkinson's disease will be required.

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