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Piperine inhibition of 1-methyl-4-phenylpyridinium-induced mitochondrial dysfunction and cell death in PC12 cells

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Abstract

The effect of alkaloid piperine against the toxicity of 1-methyl-4-phenylpyridinium (MPP^+) in differentiated PC12 cells was assessed. Piperine treatment revealed a differential effect on the cytotoxicity of MPP^+ and had its maximum inhibitory effect at 1 μM. The addition of piperine (0.5–10 μM) significantly reduced the MPP^+-induced nuclear damage, mitochondrial membrane permeability changes, formation of reactive oxygen species and depletion of GSH. In contrast, piperine at 50–100 μM showed cytotoxicity and exhibited an additive effect against the MPP^+ toxicity. The results indicate that piperine had a differential effect on the cytotoxicity of MPP^+ depending on concentration. Piperine at low concentrations may reduce the MPP^+-induced viability loss in PC12 cells by suppressing the changes in the mitochondrial membrane permeability, leading to the release of cytochrome *c* and subsequent activation of caspase-3. The effects may be ascribed to its inhibitory action on the formation of reactive oxygen species and depletion of GSH.

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

1. Introduction

It has been shown that the membrane permeability transition of mitochondria is involved in a variety of toxic and oxidative forms of cell injury as well as apoptosis (Crompton, 1999). Much evidence indicates that formation of the mitochondrial permeability transition plays a critical role in the cytotoxicity of neurotoxin 1-methyl-4-phenylpyridinium (MPP^+). Neuronal cell death due to MPP^+ is mediated by opening of the mitochondrial permeability transition pore, release of cytochrome *c*, and activation of caspases (Cassarino et al., 1999; Lotharius et al., 1999; Lee et al., 2000, 2005b). The implication of oxidative stress in the cytotoxicity has been demonstrated by that infusion of MPP^+ into the brains of mice and rats increases the formations of lipid peroxides and hydroxyl radicals in the striatum (Rojas and Rios, 1993; Obata, 2002). In contrast, MPP^+ does not induce lipid peroxidation in PC12 cells and antioxidants do not prevent the MPP^+-induced decrease in [^{3}H] dopamine uptake in cells (Fonck and Baudry, 2001). It is therefore uncertain whether the cytotoxicity of MPP^+ is mediated by increased formation of reactive oxygen species.

Piperine is an alkaloid found naturally in plants belonging to the Piperacese family, such as *Piper nigrum* Linn, known as black pepper, and *Piper longum* Linn, known as long pepper. Piperine has been used extensively as a condiment and flavoring for all types of savory dishes (Govindarajan, 1977). Piper species have been used in folklore medicine for the treatment of various diseases, including seizure disorders (Pei, 1983; Singh, 1992; D'Hooge et al., 1996). Piperine is suggested to have anti-anxiety effect, central depression and anti-inflammatory activity (Stohr et al., 2001; Singh and Singh, 2002). Mitochondrial monoamine oxidase (MAO)-B is considered to play a part in the progress of nigrostriatal cell death. It has been proposed that *R*-(−)-deprenyl exerts a beneficial effect in the treatment of Parkinson's disease through a selective inhibition of MAO-B (Birkmayer et al., 1985). Piperine shows an inhibitory effect

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against MAO-A and B (Kong et al., 2004; Lee et al., 2005c). This alkaloid is demonstrated to have antioxidant ability (Mittal and Gupta, 2000) and reduces the benzo(*a*)pyrene-induced oxidative stress in lung tissues (Selvendiran et al., 2004, 2005a,b).

In contrast to these reports, it has been shown that piperine promotes the benzo(*a*)pyrene-induced DNA damage and cell death in cultured V-79 lung fibroblast cells (Chu et al., 1994). Piperine exhibits a toxic effect against cultured hippocampal neurons (Unchern et al., 1997). Meanwhile, it has been demonstrated that piperine shows a differential damaging effect on lipid peroxidation and thiol status in intestinal tissues (Khajuria et al., 1999). Therefore, it uncertain that piperine itself exerts a cyto-protective effect or not. In addition, the effect of piperine against toxic or oxidative neuronal cell injury has not been clarified. Furthermore, with respect to mitochondrial function, the understanding of the action of piperine remains to be uncertain. Therefore, the aim of the present study was to investigate the effect of alkaloid piperine (obtained from Sigma-Aldrich Inc.) on the cytotoxicity of MPP^+ in differentiated PC12 cells in relation to changes in the mitochondrial membrane permeability.

2. Materials and methods

2.1. Materials

Wizard® Genomic DNA purification kit was purchased from Promega Co. (Madison, WI), Quantikine® M rat/mouse cytochrome *c* assay kit was from R&D systems (Minneapolis, MN), anti-mouse Bax monoclonal antibody and anti-mouse Bcl-2 monoclonal antibody were from Trevigen Inc. (Gaithersburg, MD), anti-cytochrome *c* (A-8) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), horseradish peroxidase-conjugated anti-mouse IgG was from EMD-Calbiochem. Co. (La Jolla, CA), SuperSignal® West Pico chemiluminescence substrate was from PIERCE Biotechnology Inc. (Rockford, IL), ApoAlert™ CPP32/caspase-3 assay kit was from CLONTECH Laboratories Inc. (Palo Alto, CA), and Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (Mn-TBAP) was from OXIS International Inc. (Portland, OR). Piperine, 1-methyl-4-phenylpyridinium (MPP^+), superoxide dismutase (SOD, from bovine erythrocytes; 2500–7000 U/mg protein), catalase (from bovine liver; 10,000–25,000 U/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 Inc. (St. Louis, MO). Protein concentration was determined by the method of Bradford according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

2.2. Cell culture

Rat PC12 cells (adrenal gland; pheochromocytoma) were obtained from Korean cell line bank (Seoul, South Korea). PC12 cells were cultured in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 U/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 (Tatton et al., 2002). Cells were washed with RPMI medium containing 1% fetal bovine serum 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 (Mosmann, 1983). 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. 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). Cell viability was expressed as a percentage of the value in control cultures.

2.4. Measurement of oligonucleosomal DNA fragmentation

The DNA fragmentation due to activation of endonucleases was assessed by gel electrophoresis. PC12 cells (1×10^6 cells/ml) were treated with MPP^+ for 24 h at 37 °C, washed with phosphate buffered saline, and DNA was isolated according to the protocol of DNA purification kit. DNA pellets were loaded onto a 1.5% agarose gel in Tris-acetate buffer, pH 8.0 and 1 mM EDTA, and separated at 100 V for 2 h. DNA fragments were visualized using a UV transilluminator after staining with ethidium bromide.

2.5. Measurement of apoptosis in cells

Apoptosis was assessed by measuring the DNA fragmentation, which occurs following the activation of endonucleases. PC12 cells (1×10^5 cells/200 μl) were treated with MPP^+ for 24 h at 37 °C, washed with phosphate buffered saline 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) (Isenberg and Klaunig, 2000). 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 ×*g* for 10 min, the supernatants were removed and the pellets suspended

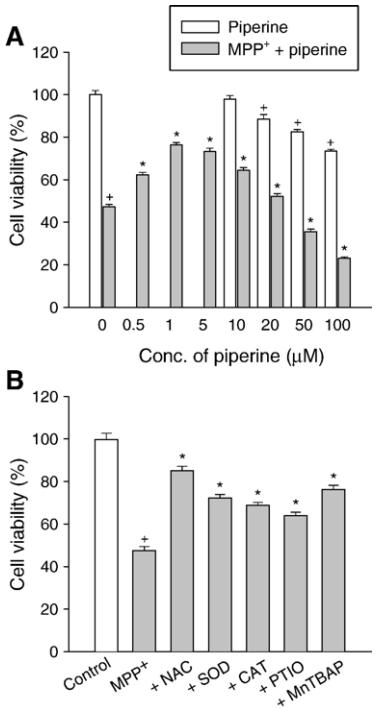

Fig. 1. Effect of piperine on MPP^+-induced cell death. PC12 cells were pretreated either with various concentrations of piperine (0.5–100 μM) in experiment A or with the scavengers (1 mM *N*-acetylcysteine [NAC], 10 μg/ml SOD, 10 μg/ml catalase [CAT], 25 μM carboxy-PTIO [PTIO] and 30 μM Mn-TBAP [MnTBAP]) in B for 30 min, exposed to 500 μM MPP^+ for 24 h, and cell viability was determined. Data represent the mean ± S.E.M. of 6–8 replicate values in two or three separate experiments. $^+P<0.05$ compared to control (percentage of control); and $^*P<0.05$ compared to MPP^+ alone.

in 1 ml of phosphate buffered saline containing 0.5 mM EDTA. For analysis, a FACScan cytofluorometer (Becton Dickinson, San Jose, CA) with argon laser excitation at 501 nm was used to assess 10,000 cells from each sample.

2.7. Western blotting analysis

PC12 cells (5×10^6 cells/ml) were harvested by centrifugation at 412 ×*g* for 10 min, washed twice with phosphate buffered saline, 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 ×*g* for 30 min and the supernatant was used for Western blotting analysis of Bax, Bcl-2 and cytochrome *c*. Supernatants were mixed with sodium dodecyl sulphate-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-Bax, anti-Bcl-2 and anti-cytochrome *c* (diluted 1:1000 in TBS containing 0.1% Tween 20) overnight at 4 °C 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 cytochrome c release

The release of cytochrome *c* from mitochondria into the cytosol was also assessed by using a solid-phase enzyme-linked immunosorbent assay kit. PC12 cells (5×10^5 cells/ml) were lysed, homogenized and the supernatant was obtained as described in Western blotting analysis. 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.

2.9. 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.

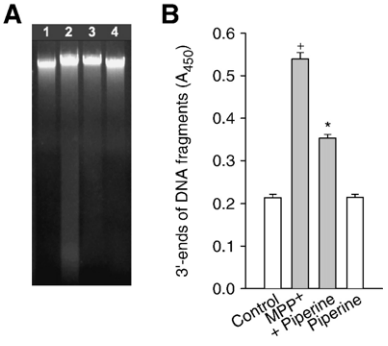

Fig. 2. Inhibition of MPP^+-induced nuclear damage by piperine. PC12 cells were treated with 500 μM MPP^+ in the presence of 1 μM piperine for 24 h. In experiment A, DNA was extracted, separated on a 1.5% agarose gel, and stained with ethidium bromide. Lane 1, untreated cells; lane 2, cells treated with MPP^+; lane 3, cells treated with MPP^+ and piperine; and lane 4, cells treated with piperine alone. In experiment B, the 3′-ends of DNA fragments were detected as described in Materials and methods. Data represent the mean ± S.E.M. of six replicate values in two separate experiments. $^+P<0.05$ compared to control; and $^*P<0.05$ compared to MPP^+ alone.

2.10. Measurement of intracellular reactive oxygen species formation

The dye DCFH2-DA, which is oxidized to fluorescent 2′,7′-dichlorofluorescin (DCF) by hydroperoxides, was used to measure relative levels of cellular peroxides (Fu et al., 1998). PC12 cells (4×10^4 cells/200 μl) were treated with MPP^+ for 24 h at 37 °C, washed, suspended in FBS-free RPMI, incubated with 50 μM dye for 30 min at 37 °C and washed with phosphate buffered saline. The cell suspensions were centrifuged at 412 ×*g* for 10 min, and medium was removed. Cells were dissolved with 1% Triton X-100 and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (SPECTRAFLUOR, TECAN, Salzburg, Austria).

2.11. Measurement of total glutathione

The total glutathione (reduced form GSH + oxidized form GSSG) was determined using glutathione reductase (van Klaveren et al., 1997). PC12 cells (4×10^4 cells/200 μl) were treated with MPP^+ for 24 h at 37 °C, centrifuged at 412 ×*g* for 10 min in a microplate centrifuge and the medium removed. The pellets were washed twice with phosphate buffered saline, dissolved with 2% 5-sulfosalicylic acid (100 μl) and incubated in 100 μl of the reaction mixture containing 22 mM sodium EDTA, 600 μM NADPH, 12 mM DTNB and 105 mM NaH_2PO_4, pH 7.5 at 37 °C. Glutathione reductase (20 μl, 100 U/ml) was added and the mixture incubated for a further 10 min. Absorbance was measured at 412 nm using a microplate reader. The standard curve was obtained from absorbance of the diluted commercial GSH that was incubated in the mixture as in samples.

2.12. Measurement of malondialdehyde

The lipid peroxidation products in PC12 cells were determined by measuring the malondialdehyde chromogen formation using thiobarbituric acid (Gutteridge et al., 1982). PC12 cells (1×10^6 cells/1 ml) were treated with MPP^+ for 24 h at 37 °C. Absorbance was measured at 532 nm. The malondialdehyde concentration was expressed as nmol of thiobarbituric acid reactive substances/milligram of protein using a molar extinction coefficient of $1.56\times10^5\ M^{-1}\ cm^{-1}$.

2.13. Statistical analysis

Data are expressed as the mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance. When significance was detected, post hoc comparisons between the different groups were made using the Duncan's test for multiple

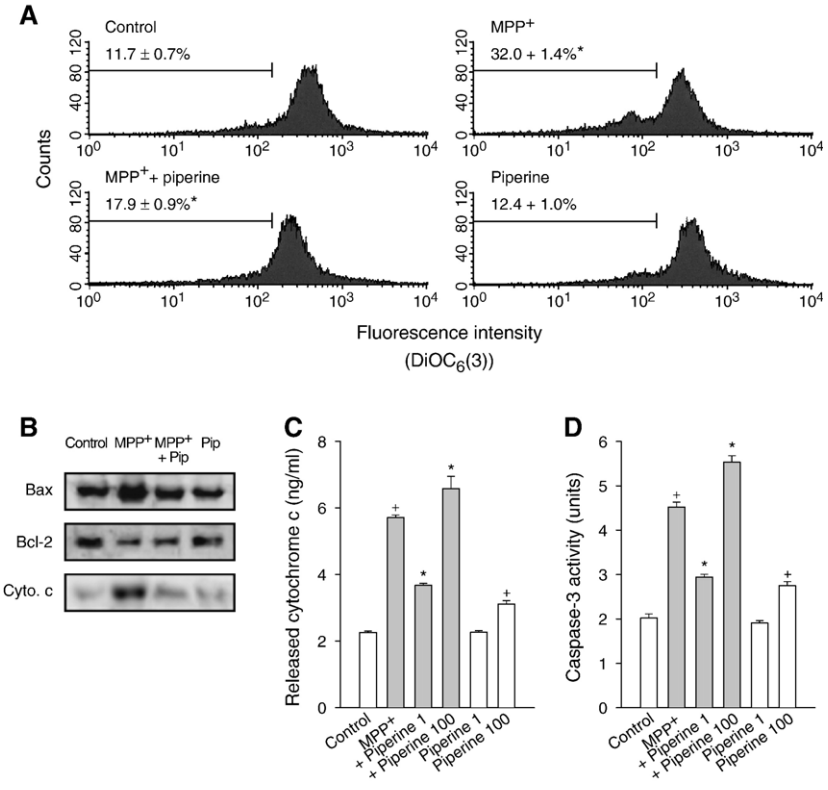

Fig. 3. Effect of piperine on MPP^+-induced loss of the mitochondrial transmembrane potential, release of cytochrome *c* and activation of caspase-3. PC12 cells were treated with 500 μM MPP^+ in the presence of 1 or 100 μM piperine (Pip, 1 μM for the Western blotting of cytochrome *c*). Data are expressed as the percentage of cells with depolarized mitochondria for the mitochondrial membrane potential (A), ng/ml for cytochrome *c* release (C) and units for caspase-3 activity (D), and represent the mean ± S.E.M. of 4–6 replicate values in two to three separate experiments. $^+P<.05$ compared to control; and $^*P<0.05$ compared to MPP^+ alone. In experiment B, the levels of Bax, Bcl-2 and cytochrome *c* (Cyto. *c*) in the cytosolic fractions were analyzed by Western blotting with anti-Bax, anti-Bcl-2 and anti-cytochrome *c* antibody. Data are representative of three different experiments.

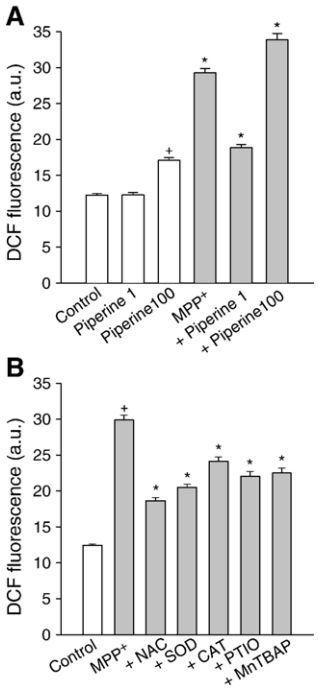

Fig. 4. Effect of piperine on MPP^+-induced reactive oxygen species formation. PC12 cells were treated either with 500 μM MPP^+ in the presence of piperine (1 or 100 μM) in experiment A or with the scavengers (1 mM *N*-acetylcysteine [NAC], 10 μg/ml SOD, 10 μg/ml catalase [CAT], 25 μM carboxy-PTIO [PTIO] and 30 μM Mn-TBAP [MnTBAP]) in B. Data are expressed as arbitrary units of fluorescence and represent the mean±S.E.M. of 6 replicate values in two separate experiments. $^+P<0.05$ compared to control; and $^*P<0.05$ compared to MPP^+ alone.

comparisons. A probability less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of piperine on MPP^+-induced cell death and nuclear damage

The effect of various concentrations of piperine on the cytotoxicity of MPP^+ was assessed in PC12 cells that are differentiated by nerve growth factor. The incidence of cell death after exposure to 500 μM MPP^+ for 24 h was about 53%. Piperine treatment revealed a differential effect on the MPP^+-induced cell death. Piperine had its maximum inhibition on cell death at 1 μM (55%); beyond this concentration the inhibitory effect declined (Fig. 1A). To assess the cytotoxic effect of piperine itself, PC12 cells were treated with the compounds in the absence of MPP^+ for 24 h. More than 20 μM, piperine itself showed a cytotoxic effect and at 100 μM it caused 27% cell viability loss. Expected from the cytotoxicity, at the concentration range of 50–100 μM piperine did not exhibit an inhibitory effect against the toxicity of MPP^+, and piperine showed an additive toxic effect.

We examined whether the toxic effect of MPP^+ against PC12 cells is mediated by the actions of reactive oxygen species and nitrogen species. Treatment with 1 mM thiol compound *N*-acetylcysteine, 10 μg/ml SOD (a superoxide scavenger), 10 μg/ml catalase (a scavenger of hydrogen peroxide), 25 μM carboxy-PTIO (a scavenger of nitric oxide) and 30 μM Mn-TBAP (a scavenger of peroxynitrite and cell-permeable metalloporphyrin that mimics SOD) reduced cell death caused by 500 μM MPP^+ (Fig. 1B).

To assess apoptotic cell death due to MPP^+ and clarify the inhibitory effect of piperine on the cytotoxicity of MPP^+, we examined nuclear damage in the MPP^+-treated cells by monitoring DNA fragmentation. During the process of apoptosis, DNA fragmentation is caused by activation of endonucleases. The effect of piperine on DNA fragmentation was assessed by agarose gel electrophoresis. DNA extracted from untreated PC12 cells (lane 1 in Fig. 2A) displayed a small increase in the oligonucleosomal cleavage of DNA. In contrast, treatment of PC12 cells with 500 μM MPP^+ displayed marked increase in DNA laddering (lane 2). Piperine (1 μM) reduced the DNA fragmentation due to MPP^+ (lane 3).

Fragmented DNA was also assessed by measuring the binding of dNTP to the 3′-ends of DNA fragments and detection by a quantitative colorimetric assay. PC12 cells were treated with 500 μM MPP^+ in the presence or absence of piperine. Control cells showed absorbance of 0.214±0.008 (mean±S.E.M. of six experiments), whilst exposure to 500 μM MPP^+ for 24 h increased the absorbance about 2.5-fold (Fig. 2B). Piperine (1 μM) significantly reduced the MPP^+-induced increase in absorbance, while absorbance in cells treated with piperine alone was not significantly different from that in control cells.

3.2. Effect of piperine on MPP^+-induced mitochondrial membrane permeability transition

To better characterize the cell death signaling events in the toxicity of MPP^+, we investigated the effect on changes in the mitochondrial membrane permeability. Changes in the mitochondrial transmembrane potential in PC12 cells treated with 500 μM MPP^+ were quantified by flow cytometry with the dye $DiOC_6(3)$. Exposure of PC12 cells to MPP^+ for 24 h increased the percentage of cells with depolarized mitochondria (characterized by low values of the transmembrane potential). Piperine

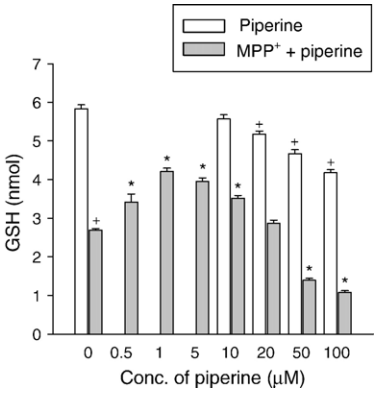

Fig. 5. Inhibition of MPP^+-induced depletion of GSH by piperine. PC12 cells were treated with 500 μM MPP^+ in the presence of piperine (0.5–100 μM). Data are expressed as nmol of GSH/mg protein and represent the mean±S.E.M. of 6 replicate values in two separate experiments. $^+P<0.05$ compared to control; and $^*P<0.05$ compared to MPP^+ alone.

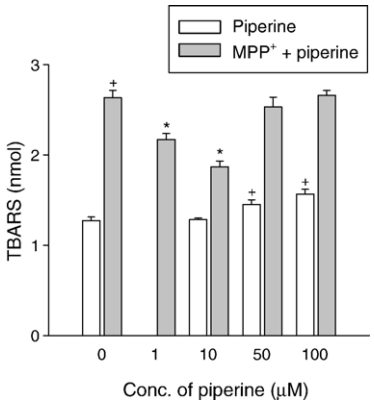

Fig. 6. Inhibition of MPP^+-induced lipid peroxidation by piperine. PC12 cells were treated with 500 μM MPP^+ in the presence of piperine (1–100 μM). Data are expressed as nmol of thiobarbituric acid reactive substances (TBARS)/mg protein and represent the mean ± S.E.M. of 6 replicate values in two separate experiments. $^+P<0.05$ compared to control; and $*P<0.05$ compared to MPP^+ alone.

(1 μM) significantly inhibited the MPP^+-induced increase in cells with depolarized mitochondria, while the mitochondrial transmembrane potential in cells treated with piperine alone was not significantly different from that in control (Fig. 3A).

We examined whether the toxicity of MPP^+ is mediated by mitochondrial apoptotic pathway using Western blotting analysis. PC12 cells exposed to MPP^+ for 24 h revealed an increase in the pro-apoptotic Bax protein and cytochrome *c* levels, and reduction in anti-apoptotic Bcl-2 level. The treatment with piperine (1 μM) reduced the effect of MPP^+-induced alterations of Bax, cytochrome *c* and Bcl-2 protein contents (Fig. 3B).

The MPP^+-induced changes in the mitochondrial membrane permeability were also confirmed by measuring a release of cytochrome *c* into the cytosol and activation of caspase-3. PC12 cells treated with 500 μM MPP^+ revealed a significant increase in the cytochrome *c* release and activation of caspase-3 activity. Piperine (1 μM) significantly depressed the MPP^+-induced release of cytochrome *c* and increase in caspase-3 activity, while piperine alone did not cause significantly the cytochrome *c* release and caspase-3 activation (Fig. 3C and D).

3.3. Effect of piperine on MPP^+-induced formation of reactive oxygen species and depletion of GSH

To determine whether the MPP^+-induced cell death is mediated by oxidative stress, we investigated the formation of reactive oxygen species within cells by monitoring a conversion of $DCFH_2$-DA to DCF. Treatment of PC12 cells with 500 μM MPP^+ resulted in a significant increase in DCF fluorescence. Piperine (1 μM) inhibited the MPP^+-induced increase in DCF fluorescence, while the compound alone did not induce changes in DCF fluorescence (Fig. 4). Piperine itself at 100 μM significantly increased the DCF fluorescence and showed an additive increasing effect on the changes in DCF fluorescence due to MPP^+.

To confirm further the formation of reactive oxygen species in PC12 cells exposed to MPP^+, we examined the inhibitory effect of antioxidants. The addition of 1 mM *N*-acetylcysteine, 10 μg/ml SOD, 10 μg/ml catalase, 25 μM carboxy-PTIO and 30 μM Mn-TBAP inhibited the increase in DCF fluorescence due to MPP^+ (Fig. 4B).

Drops in cellular GSH levels increase the sensitivity of neurons to the toxic effect of neurotoxins and are associated with mitochondrial dysfunction (Chandra et al., 2000). The work conducted whether the inhibitory effect of piperine on the toxicity of MPP^+ was ascribed to the effect on the depletion of GSH. The thiol content in the control PC12 cells was 5.84 ± 0.10 nmol/mg protein. Treatment with 500 μM MPP^+ for 24 h depleted GSH contents by 54%. Piperine (0.5–10 μM) significantly inhibited the MPP^+-induced depletion of GSH in PC12 cells and showed a maximum inhibition at 1 μM (Fig. 5). Piperine at the concentration range of 50–100 μM reduced the GSH contents and exhibited an additive decreasing effect on the MPP^+-induced reduction in the GSH content.

To examine whether the inhibitory effect of piperine on the toxicity of MPP^+ is mediated by the antioxidant ability, we assessed the effect of piperine on lipid peroxidation in PC12 cells exposed to MPP^+. Treatment of PC12 cells with 500 μM MPP^+ for 24 h produced 1.361 nmol/mg protein of thiobarbituric acid reactive substances (Fig. 6). Piperine at the concentration range of 1–10 μM attenuated the MPP^+-lipid peroxidation in PC12 cells. Meanwhile piperine at 50–100 μM induced lipid peroxidation and did not attenuate the oxidizing effect of MPP^+.

4. Discussion

The cytotoxic effect of MPP^+ on differentiated PC12 cells was assessed by measuring the nuclear damage, mitochondrial dysfunction and cell death. PC12 cells upon the nerve growth factor stimulation not only display abundant neuritic growth, but also adopt a neurochemical dopaminergic phenotype (Kadota et al., 1996). Formation of the mitochondrial permeability transition causes a release of cytochrome *c* from mitochondria and subsequent activation of caspase-3 that is involved in apoptotic cell death (Crompton, 1999). The MPP^+ treatment elicits the respiratory chain inhibition, leading to the formation of free radicals, and the activation of the mitochondrial permeability transition (Obata, 2002; Jenner, 2003). The MPP^+-induced apoptosis in neuronal cells is mediated by loss of the mitochondrial transmembrane potential, which results in the release of mitochondrial cytochrome *c* and subsequent activation of caspase-3 (Cassarino et al., 1999; Lee et al., 2005b). Consistent with this finding, in this study the MPP^+-induced apoptotic cell death in differentiated PC12 cells was demonstrated by the condensation and fragmentation of nuclei and by the caspase-3 activation. In agreement with the previous reports (Dennis and Bennett, 2003; Youdim and Arraf, 2004), the toxicity of MPP^+ via mitochondrial pathway was demonstrated by the increase in the levels of pro-apoptotic Bax protein and cytochrome *c* and by the decrease in anti-apoptotic Bcl-2 levels. The mitochondrial membrane permeability transition could induce formation of reactive oxygen species and nitrogen species by inhibition of respiratory chain and vice versa (Fleury et al., 2002; Polster and Fiskum, 2004). The formation of reactive oxygen species in PC12 cells exposed to MPP^+ and the inhibitory effect of antioxidants, including Mn-

TBAP and carboxy-PTIO, suggest that MPP^+ induces the formation of reactive oxygen species and nitrogen species in PC12 cells, leading to mitochondrial dysfunction. Meanwhile, the inhibitory effect of SOD and catalase indicates that the cytotoxicity of MPP^+ seems to be mediated by extracellulary released-superoxide and hydrogen peroxide.

It has been shown that piperine has anti-oxidant and anti-inflammatory activity (Mittal and Gupta, 2000; Stohr et al., 2001). However, piperine does not show a consistent effect on the toxicity of benzo(*a*)pyrene in lung tissues and cultured cell line (Chu et al., 1994; Selvendiran et al., 2004). In addition, it is demonstrated that piperine itself exhibits a differential cytotoxic effect depending on cell type (Unchern et al., 1997). Under the contradictory findings, therefore, one of the aims of this study was to assess the effect of various concentrations of piperine against cell viability in PC12 cells. Piperine reduced the cytotoxicity of MPP^+ with a maximal inhibition at 1 μM; beyond these concentrations the inhibitory effect declined. The cytotoxic effect of piperine itself was observed at the concentrations more than 20 μM, and it showed an additive effect on the toxicity of MPP^+. From these findings, piperine seems to exhibit a differential protective effect on the toxic neuronal cell injury depending on the concentration.

The current data clearly indicate the inhibitory effect of piperine at low concentrations against the cytotoxicity of MPP^+. Piperine affects mitochondrial enzymes involved in energy metabolism (Jamwal and Singh, 1993). The attenuation of benzo(*a*)pyrene-induced lung carcinogenesis by piperine may be ascribed to its stimulatory effect on mitochondrial TCA cycle enzymes and glutathione-metabolizing enzyme (Selvendiran et al., 2005b). However, it is uncertain that the preventive effect of piperine comes from its inhibitory action on the mitochondrial permeability transition. Therefore, with respect to the mitochondrial membrane permeability we examined the effect of piperine against the cytotoxicity of MPP^+. The present data suggest that piperine at the low concentrations reduces the MPP^+-induced cell death in differentiated PC12 cells by suppressing the loss of mitochondrial transmembrane potential, increase in Bax protein level, cytochrome *c* release and subsequent caspase-3 activation and by increasing Bcl-2 protein. Piperine is suggested to attenuate oxidative stress in the toxicity of carcinogen (Selvendiran et al., 2004). The report and the current data indicate that the inhibitory effect of piperine against the MPP^+-induced mitochondrial damage seems to partly be ascribed to its anti-oxidant action. Meanwhile, the cytotoxicity of piperine itself at high concentrations also seems to be linked to the formation of the mitochondrial membrane permeability transition, and the additive effect of piperine on the MPP^+-induced cell death may come from mitochondrial dysfunction.

Depletion of cellular and mitochondrial GSH is demonstrated to increase the formation of reactive oxygen species, and the oxidation and depletion of GSH induce the formation of the mitochondrial membrane permeability transition (Constantini et al., 1996; Chandra et al., 2000; Lee et al., 2005a). During the apoptotic process, drops in GSH levels and concomitant increase in reactive oxygen species are detected (Tan et al., 1998). It has been shown that piperine caused cell injury in rat intestinal tissues by inducing lipid peroxidation, in which however the level of GSH and protein thiols was not altered rather increased (Khajuria et al., 1999). In contrast to this report, the present finding indicates that high concentrations of piperine-induced mitochondrial dysfunction may be associated with the formation of reactive oxygen species, decrease in the GSH contents and increase in lipid peroxidation. The inhibitory effect of piperine at low concentrations on the MPP^+-induced cell death approximately correlated with the effect on GSH depletion. The current data suggest that piperine at the low concentrations seems to reduce the MPP^+-induced changes in the mitochondrial membrane permeability by suppressing oxidative stress and depletion of cellular GSH.

Overall, the results presented here indicate that piperine reveals a differential effect against the cytotoxicity of MPP^+ depending on concentration. Piperine at low concentrations may reduce the MPP^+-induced viability loss in PC12 cells by suppressing the mitochondrial membrane permeability transition, leading to the activation of caspase-3. The effect seems to be associated with the decrease in the reactive oxygen species formation and in the depletion of GSH.

References

Birkmayer, W., Knoll, J., Riederer, P., Youdim, M.B., Hars, V., Marton, J., 1985. Increased life expectancy resulting from addition of L-deprenyl to Madopar treatment in Parkinson's disease: a longterm study. J. Neural Transm. 64, 113–127.

Cassarino, D.S., Parks, J.K., Parker Jr., W.D., Bennett Jr., J.P., 1999. The parkinsonian neurotoxin MPP^+ opens the mitochondrial permeability transition pore and releases cytochrome *c* in isolated mitochondria via an oxidative mechanism. Biochim. Biophys. Acta 1453, 49–62.

Chandra, J., Samali, A., Orrenius, S., 2000. Triggering and modulation of apoptosis by oxidative stress. Free Radic. Biol. Med. 29, 323–333.

Chu, C.Y., Chang, J.P., Wang, C.J., 1994. Modulatory effect of piperine on benzopyrene cytotoxicity and DNA adduct formation in V-79 lung fibroblast cells. Food Chem. Toxicol. 32, 373–377.

Constantini, P.C., Chernyak, B.C., Petronilli, V., Bernardi, P., 1996. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. J. Biochem. Biol. 271, 6746–6751.

Crompton, M., 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 341, 233–249.

Dennis, J., Bennett Jr., J.P., 2003. Interactions among nitric oxide and Bcl-family proteins after MPP^+ exposure of SH-SY5Y neural cells I: MPP^+ increases mitochondrial NO and Bax protein. J. Neurosci. Res. 72, 76–88.

D'Hooge, R., Pei, Y.Q., Raes, A., Lebrun, P., van Bogaert, P.P., de Deyn, P.P., 1996. Anticonvulsant activity of piperine on seizures induced by excitatory amino acid receptor agonists. Artzneim. Forsch. 46, 557–560.

Fleury, C., Mignotte, B., Vayssiere, J.-L., 2002. Mitochondrial reactive oxygen species in cell death signaling. Biochimie 84, 131–141.

Fonck, C., Baudry, M., 2001. Toxic effects of MPP^+ and MPTP in PC12 cells independent of reactive oxygen species formation. Brain Res. 905, 199–206.

Fu, W., Luo, H., Parthasarathy, S., Mattson, M.P., 1998. Catecholamines potentiate amyloid β-peptide neurotoxicity: involvement of oxidative stress, mitochondrial dysfunction, and perturbed calcium homeostasis. Neurobiol. Dis. 5, 229–243.

Govindarajan, V.S., 1977. Pepper chemistry, technology and quality evaluation. Crit. Rev. Food Sci. Nutr. 9, 115–125.

Gutteridge, J.M.C., Rowley, D.A., Halliwell, B., 1982. Superoxide dependent formation of hydroxyl radicals and lipid peroxidation in the presence of iron salts. Detection of 'catalytic' iron and anti-oxidant activity in extracellular fluids. Biochem. J. 201, 605–609.

Isenberg, J.S., Klaunig, J.E., 2000. Role of the mitochondrial membrane permeability transition (MPT) in rotenone-induced apoptosis in liver cells. Toxicol. Sci. 53, 340–351.

Jamwal, D.S., Singh, J., 1993. Effects of piperine on enzyme activities and bioenergetic functions in isolated rat liver mitochondria and hepatocytes. J. Biochem. Toxicol. 8, 167–174.

Jenner, P., 2003. Oxidative stress in Parkinson's disease. Ann. Neurol. 53 (suppl 3), S26–S38.

Kadota, T., Yamaai, T., Saito, Y., Akita, Y., Kawashima, S., Moroi, K., Inagaki, N., Kadota, K., 1996. Expression of dopamine transporter at the tips of growing neurites of PC12 cells. J. Histochem. Cytochem. 44, 989–996.

Khajuria, A., Johrn, R.K., Zutshi, U., 1999. Piperine mediated alterations in lipid peroxidation and cellular thiol status of rat intestinal mucosa and epithelial cells. Phytomedicine 6, 351–355.

Kong, L.D., Cheng, C.H., Tan, R.X., 2004. Inhibition of MAO A and B by some plant-derived alkaloids, phenols and anthraquinones. J. Ethnopharmacol. 91, 351–355.

Lee, C.S., Han, E.S., Jang, Y.Y., Han, J.H., Ha, H.W., Kim, D.E., 2000. Protective effect of harmalol and harmaline on MPTP neurotoxicity in the mouse and dopamine-induced damage of brain mitochondria and PC12 Cells. J. Neurochem. 75, 521–531.

Lee, C.S., Kim, Y.J., Ko, H.H., Han, E.S., 2005a. Synergistic effects of hydrogen peroxide and ethanol on cell viability loss in PC12 cells by increase in mitochondrial permeability transition. Biochem. Pharmacol. 70, 317–325.

Lee, C.S., Park, S.Y., Ko, H.H., Song, J.H., Shin, Y.K., Han, E.S., 2005b. Inhibition of MPP^+-induced mitochondrial damage and cell death by trifluoperazine and W-7 in PC12 cells. Neurochem. Int. 46, 169–178.

Lee, S.A., Hong, S.S., Han, X.B., Hwang, J.S., Oh, G.J., Lee, K.S., Lee, M.K., Hwang, B.Y., Ro, J.S., 2005. Piperine from the fruits of *Piper longum* with inhibitory effect on monoamine oxidase and antidepressant-like activity. Chem. Pharm. Bull. 53, 832–835.

Lotharius, J., Dugan, L.L., O'Malley, K.L., 1999. Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. J. Neurosci. 19, 1284–1293.

Mittal, R., Gupta, R.L., 2000. In vitro antioxidant activity of piperine. Methods Find. Exp. Clin. Pharmacol. 22, 271–274.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Obata, T., 2002. Dopamine efflux by MPTP and hydroxyl radical generation. J. Neural. Transm. 109, 1159–1180.

Pei, Y.Q., 1983. A review of pharmacology and clinical use of piperine and its derivatives. Epilepsia 24, 177–182.

Polster, B.M., Fiskum, G., 2004. Mitochondrial mechanisms of neural cell apoptosis. J. Neurochem. 90, 1281–1289.

Rojas, P., Rios, C., 1993. Increased striatal lipid peroxidation after intracerebroventricular MPP^+ administration to mice. Pharmacol. Toxicol. 72, 364–368.

Selvendiran, K., Banu, S.M., Sakthisekaran, D., 2004. Protective effect of piperine on benzo(*a*)pyrene-induced lung carcinogenesis in Swiss albino mice. Clin. Chim. Acta 350, 73–78.

Selvendiran, K., Banu, S.M., Sakthisekaran, D., 2005a. Oral supplement of piperine leads to altered phase II enzymes and reduced DNA damage and DNA–protein cross links in benzo(*a*)pyrene induced experimental lung carcinogenesis. Mol. Cell. Biochem. 268, 141–147.

Selvendiran, K., Thirunavukkarasu, C., Prince Vijeya Singh, J., Padmavathi, R., Sakthisekaran, D., 2005b. Chemopreventive effect of piperine on mitochondrial TCA cycle and phase-I and glutathione-metabolizing enzymes in benzo(*a*)pyrene induced lung carcinogenesis in Swiss albino mice. Mol. Cell. Biochem. 271, 106–111.

Singh, Y.N., 1992. Kava: an overview. J. Ethnopharmacol. 37, 13–45.

Singh, Y.N., Singh, N.N., 2002. Therapeutic potential of kava in the treatment of anxiety disorders. CNS Drugs 16, 731–743.

Stohr, J.R., Xiaso, P.G., Bauer, R., 2001. Constituents of Chinese piper species and their inhibitory activity on prostaglandin and leukotriene biosynthesis in vitro. J. Ethnopharmacol. 75, 133–139.

Tan, S., Sagara, Y., Liu, Y., Maher, P., Schubert, D., 1998. The regulation of reactive oxygen species production during programmed cell death. J. Cell Biol. 141, 1423–1432.

Tatton, W.G., Chalmers-Redman, R.M.E., Ju, W.J.H., Mammen, M., Carlile, G.W., Pong, A.W., Tatton, N.A., 2002. Propargylamines induce antiapoptotic new protein synthesis in serum-and nerve growth factor (NGF)-withdrawn, NGF-differentiated PC-12 cells. J. Pharmacol. Exp. Ther. 301, 753–764.

Unchern, S., Saito, H., Nishiyama, N., 1997. Selective cytotoxicity of piperine on cultured rat hippocampal neurons in comparison with cultured astrocytes: the possible involvement of lipid peroxidation. Biol. Pharm. Bull. 20, 958–961.

van Klaveren, R.J., Hoet, P.H.M., Pype, J.L., Demedts, M., Nemery, B., 1997. Increase in γ-glutamyltransferase by glutathione depletion in rat type II pneumocytes. Free Radic. Biol. Med. 22, 525–534.

Youdim, M.B., Arraf, Z., 2004. Prevention of MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) dopaminergic neurotoxicity in mice by chronic lithium: involvements of Bcl-2 and Bax. Neuropharmacology 46, 1130–1140.