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Promoting Effect of Hydrogen Peroxide on 1-Methyl-4-phenylpyridinium-induced Mitochondrial Dysfunction and Cell Death in PC12 Cells

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The promoting effect of hydrogen peroxide (H_2O_2) against the cytotoxicity of 1-methyl-4-phenylpyridinium (MPP^+) in differentiated PC12 cells was assessed by measuring the effect on the mitochondrial membrane permeability. Treatment of PC12 cells with MPP^+ resulted in the nuclear damage, decrease in the mitochondrial transmembrane potential, cytosolic accumulation of cytochrome c, activation of caspase-3, increase in the formation of reactive oxygen species (ROS) and depletion of GSH. Addition of H_2O_2 enhanced the MPP^+-induced nuclear damage and cell death. Catalase, Carboxy-PTIO, Mn-TBAP, *N*-acetylcysteine, cyclosporin A and trifluoperazine inhibited the cytotoxic effect of MPP^+ in the presence of H_2O_2. Addition of H_2O_2 promoted the change in the mitochondrial membrane permeability, ROS formation and decrease in GSH contents due to MPP^+ in PC12 cells. The results show that the H_2O_2 treatment promotes the cytotoxicity of MPP^+ against PC12 cells. H_2O_2 may enhance the MPP^+-induced viability loss in PC12 cells by promoting the mitochondrial membrane permeability change, release of cytochrome c and subsequent activation of caspase-3, which is associated with the increased formation of ROS and depletion of GSH. The findings suggest that H_2O_2 as a promoting agent for the formation of mitochondrial permeability transition may enhance the neuronal cell injury caused by neurotoxins.

Key Words: 1-Methyl-4-phenylpyridinium, H_2O_2, PC12 cells, Mitochondrial membrane permeability, Cell injury

INTRODUCTION

The membrane permeability transition of mitochondria is known as a central event in the course of a variety of toxic and oxidative forms of cell injury as well as apoptosis (Cassarino et al, 1999; Crompton, 1999). Opening of the mitochondrial permeability transition pore causes a depolarization of the transmembrane potential, releases of Ca^{2+} and cytochrome c, osmotic swelling and loss of oxidative phosphorylation. The permeability transition pore is suggested as target of the dopamine oxidation products and 1-methyl-4-phenylpyridinium (MPP^+) (Berman & Hastings, 1999; Lee et al, 2002, 2003). Neuronal cell death due to MPP^+ is mediated by opening of the mitochondrial permeability transition pore, releases of Ca^{2+} and cytochrome c, and activation of caspases (Cassarino et al, 1999; Lotharius et al, 1999). Infusion of MPP^+ into the brains of mice and rats increases the formations of lipid peroxides and hydroxyl radicals in the striatum (Rojas & Rios, 1993; Obata et al, 2003). Meanwhile, the MPP^+-induced decrease in [^{3}H] dopamine uptake in PC12 cells is not prevented by GSH and superoxide scavengers (Fonck & Baudry, 2001). In this study, MPP^+ does not induce lipid peroxidation in PC12 cells. It is therefore uncertain whether reactive oxygen species (ROS) is involved in cytotoxicity of MPP^+.

The oxidation of dopamine liberates free radicals and dopamine quinone, which cause a swelling of isolated brain mitochondria and loss of the mitochondrial transmembrane potential. MPP^+ is demonstrated to stimulate the displacement of dopamine from vesicular storage sites to the cytoplasm, which further induces an oxidation of dopamine (Lotharius & O'Malley, 2000). The co-addition of dopamine and MPP^+ shows an enhancing effect on the mitochondrial membrane permeability change and cell death (Lee et al, 2003). Neuronal cell death due to mitochondrial complex I inhibitors and MPP^+ is mediated by the opening of the mitochondrial permeability pore and the collapse of the mitochondrial transmembrane potential (Lotharius et al, 1999; Lee et al, 2002).

Hydrogen peroxide, one of the products of dopamine oxidation, diffuses partly into the mitochondrial matrix and

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ABBREVIATIONS: carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; $DiOC_6(3)$, 3,3'-dihexyloxacarbocyanine iodide; $DCFH_2$-DA, 2',7'-dichlorofluorescin diacetate; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); Mn-TBAP, Mn (III) tetrakis (4-benzoic acid) porphyrin chloride (Mn-TBAP); MPP^+, 1-methyl-4-phenylpyridinium; PMSF, phenylmethylsulfonylfluoride; ROS, reactive oxygen species.

oxidizes GSH to glutathione disulphide, which is thought to induce the alteration of cellular functions, including suppression of the thiol-dependent electron transport (Cohen et al, 1997). Inhibition of the mitochondrial respiratory chain enhances superoxide formation that can initiate apoptotic cell death through a decrease in the mitochondrial membrane potential (Fleury et al, 2002; Jenner, 2003). It has been shown that the hydrogen peroxide-induced cell death is mediated by mitochondrial damage and cytochrome c, which accompanied by the activation of caspase-3 and -9 (Mronga et al, 2004), by upregulation of the Fas receptor/ligand system (Cabaner et al, 1999) and by disruption of the intracellular Ca^{2+} homeostasis (Wang & Joseph, 2000). The sub-lethal doses of tumor necrosis factor- and hydrogen peroxide show a combined toxic effect on PC12 cells (Trembovler et al, 2003).

MPP^+ is suggested to reveal oxidative forms of neuronal cell injury through the induction of the membrane permeability transition. MPP^+ seems to exhibit a toxic effect on neuronal cell by inducing of dopamine oxidation. However, the effect of H_2O_2 on the cytotoxicity of MPP^+ has not been elucidated. To elucidate the action of H_2O_2 as a promoting agent for oxidative cell injury, the present study was performed to assess the effect of H_2O_2 on differentiated PC12 cells against the cytotoxicity of MPP^+ in relation to change in the mitochondrial membrane permeability.

METHODS

Materials

TiterTACS™ colorimetric apoptosis detection kit was purchased from Trevigen, Inc. (Gaithersburg, MD, USA), Quantikine® M rat/mouse cytochrome c assay kit was from R&D systems (Minneapolis, MN, USA), ApoAlert™ CPP32/caspase-3 assay kit was from CLONTECH Laboratories Inc. (Palo Alto, CA, USA), and Mn (III) tetrakis (4-benzoic acid)porphyrin chloride (Mn-TBAP) was from OXIS International Inc. (Portland, OR, USA). 1-Methyl-4-phenylpyridinium (MPP^+), catalase (from bovine liver; 10,000-25,000 units/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 Co. (St. Louis, MO, USA). Protein concentration was determined by the method of Bradford according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

Cell culture

Rat PC12 cells (adrenal gland; pheochromocytoma) were obtained from Korean cell line bank (Seoul, Korea). PC12 cells were cultured in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS), 100 units/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% FBS 24 h before experiments and replated onto the 96- and 24-well plates.

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^+ and H_2O_2 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, USA). Cell viability was expressed as a percentage of the value in control cultures.

Morphological observation of nuclear change

PC12 cells (2×10^5 cells/ml) were treated with MPP^+ for 24 h at 37°C and the nuclear morphological change was assessed using the Hoechst dye 33258 (Oberhammer et al, 1992). Cells were washed 1 ml phosphate-buffered saline (PBS) and incubated with 1 μg/ml Hoechst 33258 for 3 min at room temperature. Nuclei were visualized using an Olympus Microscope with a WU excitation filter (Tokyo, Japan).

Measurement of apoptosis in cells

Apoptosis was assessed by measuring the DNA fragmentation, which occurs following the activation of endonucleases. PC12 cells (4×10^4 cells/200 μl) were treated with MPP^+ for 24 h at 37°C, washed with PBS 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.

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 & Klaunig, 2000). Cells (1×10^6 cells/ml) were treated with H_2O_2 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 in 1 ml of PBS containing 0.5 mM EDTA. For analysis, a FACScan cytofluorometer (Becton Dickinson, San Jose, CA, USA) with argon laser excitation at 501 nm was used to assess 10,000 cells from each sample.

Measurement of cytochrome c release

The release of cytochrome c from mitochondria into the cytosol was assessed by using a solid-phase enzyme-linked immunosorbent assay kit for the detection of cytochrome c. PC12 cells (5×10^5 cells/ml) were harvested by centrifugation at 412×g for 10 min, washed twice with PBS, 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 analysis of cytochrome c. 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.

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.

Measurement of intracellular ROS formation

The dye $DCFH_2$-DA, which is oxidized to fluorescent 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 M dye for 30 min at 37°C and washed with PBS. 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).

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 PBS, 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 units/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.

Statistical analysis

Data are expressed as means±SEM. 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 comparisons. A probability less than 0.05 was considered to be statistically significant.

RESULTS

H_2O_2 enhances MPP^+-induced cell death and nuclear damage

The effect of various concentrations of H_2O_2 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 130 μM MPP^+ for 24 h was about 16%. H_2O_2 (10~75 μM) significantly enhanced the 130 μM MPP^+-induced cell death in a dose-dependent manner and the cytotoxic effect of MPP^+ plus H_2O_2 was apparently greater than the sum of that of each compound (Fig. 1). To assess the cytotoxic effect of H_2O_2 itself, PC12 cells were treated with H_2O_2 in the absence of MPP^+ for 24 h. Treatment with H_2O_2 (10~75 μM) caused 5~45% cell viability loss.

We examined whether the toxic effect of MPP^+ and H_2O_2 against PC12 cells is mediated by the actions of ROS and nitrogen species. Treatment with 1 mM thiol compound *N*-acetylcysteine 10 μg/ml superoxide dismutase (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 superoxide dismutase) cell death caused either by 130 μM MPP^+ plus 75 μM H_2O_2 or by H_2O_2 alone (Fig. 2).

To assess apoptotic cell death due to MPP^+ and clarify the stimulatory effect of H_2O_2 against the cytotoxicity of MPP^+, we investigated the effect of H_2O_2 on the nuclear morphological changes observed in the MPP^+-treated cells.

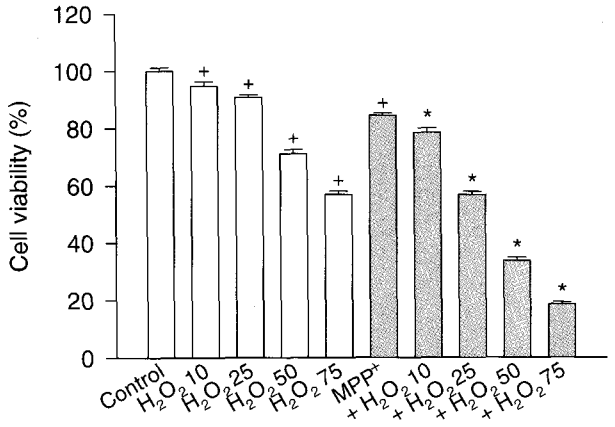

Fig. 1. Effect of H_2O_2 on MPP^+-induced cell death. PC12 cells were pre-treated with H_2O_2 (10~75 μM) for 30 min, exposed to 130 μM MPP^+ for 24 h, and cell viability was determined. Data represent means±SEM (n=6). $^+P<0.05$ compared to control (percentage of control); and $^*P<0.05$ compared to MPP^+ alone.

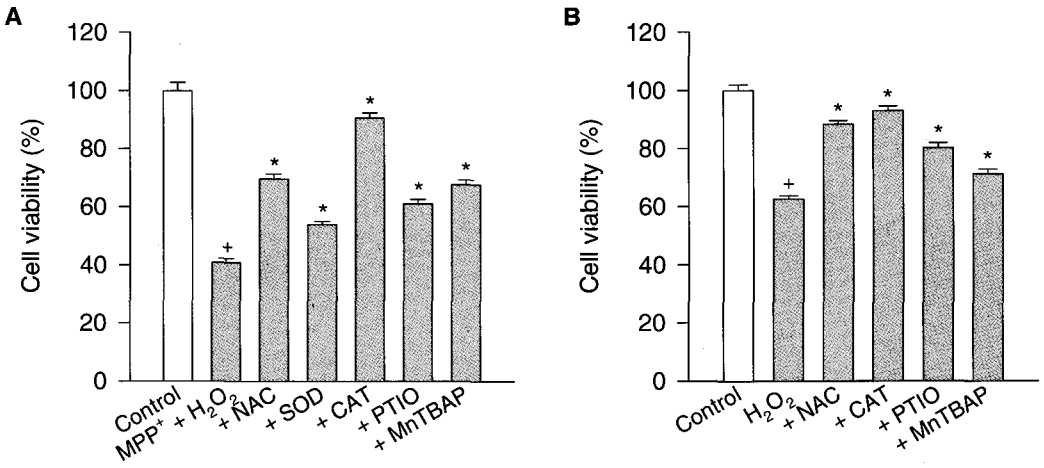

Fig. 2. Effect of antioxidants on MPP^+ and H_2O_2-induced cell death. PC12 cells were pre-treated with the scavengers [10 μg/ml SOD, 10 μg/ml catalase (CAT), 25 μM carboxy-PTIO (PTIO), 30 μM Mn-TBAP (MnTBAP), 1 mM *N*-acetylcysteine (NAC)] for 30 min, and then exposed either to 130 μM MPP^+ plus H_2O_2 (A) or to H_2O_2 alone (B) for 24 h, and cell viability was determined. Data represent means±SEM (n=6). $^+P<0.05$ compared to control; $^*P<0.05$ compared to either MPP^+ plus H_2O_2 or H_2O_2 alone. H_2O_2 was expressed as HP, and MPP^+ plus H_2O_2 as MH.

Nuclear staining with Hoechst 33258 demonstrated that control PC12 cells had regular and round-shaped nuclei. In contrast, the condensation and fragmentation of nuclei, characteristic of apoptotic cells, were evident in cells treated with 130 μM MPP^+ and 75 μM H_2O_2 (Fig. 3A). N-Acetylcysteine (1 mM) depressed the MPP^+ plus H_2O_2-induced nuclear damage.

During the process of apoptosis, DNA fragmentation is caused by activation of endonucleases. Fragmented DNA was 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 130 μM MPP^+ in the presence or absence of H_2O_2. Control cells showed absorbance of 0.215±0.006 (means±SEM of six experiments), whilst exposure to 130 μM MPP^+ for 24 h increased the absorbance about 17% (Fig. 3B). Despite the damaging effect, H_2O_2 (25~75 μM) significantly enhanced the fragmentation of DNA due to MPP^+. The increase in absorbance due to co-addition of MPP^+ and H_2O_2 was greater than the sum of that of each compound.

H_2O_2 enhances MPP^+-induced changes in mitochondrial membrane permeability

We assessed the cytotoxic effect of MPP^+ by investigating its effect on the mitochondrial membrane permeability. Change in the mitochondrial transmembrane potential in PC12 cells treated with 130 μM MPP^+ was quantified by flow cytometry with the dye $DiOC_6(3)$. Addition of 75 μM H_2O_2 significantly enhanced the MPP^+-induced increase in cells with depolarized mitochondria (characterized by low values of the transmembrane potential) (Fig. 4A). Increase in the percentage of cells with depolarized mitochondria in exposed to MPP^+ plus H_2O_2 was greater than the sum of that of each compound.

The MPP^+-induced change in the mitochondrial membrane permeability was assessed by measuring a release of cytochrome c into the cytosol and subsequent activation of caspase-3. PC12 cells treated with 130 μM MPP^+ showed

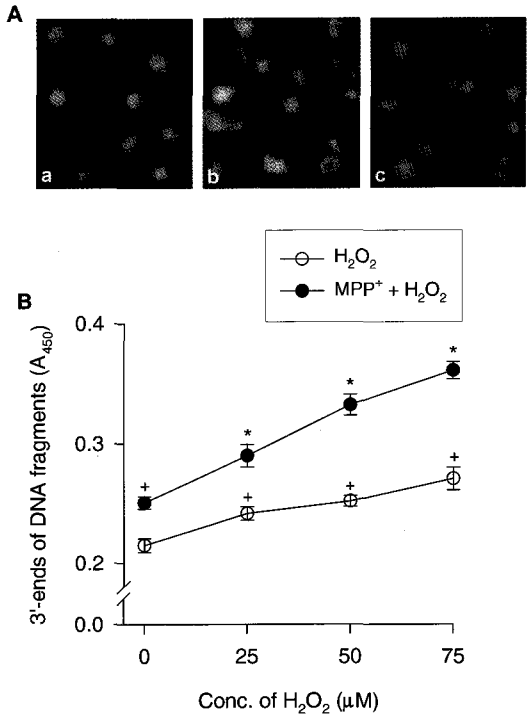

Fig. 3. Enhancement of MPP^+- induced nuclear damage by H_2O_2. PC12 cells were treated with 130 μM MPP^+ in the presence of 25~75 μM H_2O_2. In experiment (A), cells were observed by fluorescence microscopy after nuclei staining with Hoechst 33258. Figure represents microscopic morphology of the control cells (a), cells treated with MPP^+ plus 75 μM H_2O_2 (b), cells treated with MPP^+ and H_2O_2 in the presence of 1 mM *N*-acetylcysteine (c). a~c are representative of four different experiments. In experiment (B), the 3'-ends of DNA fragments were detected as described in Methods. Data are expressed as absorbance and represent means±SEM (n=6). $^+P<0.05$ compared to control; and $^*P<0.05$ compared to MPP^+ alone.

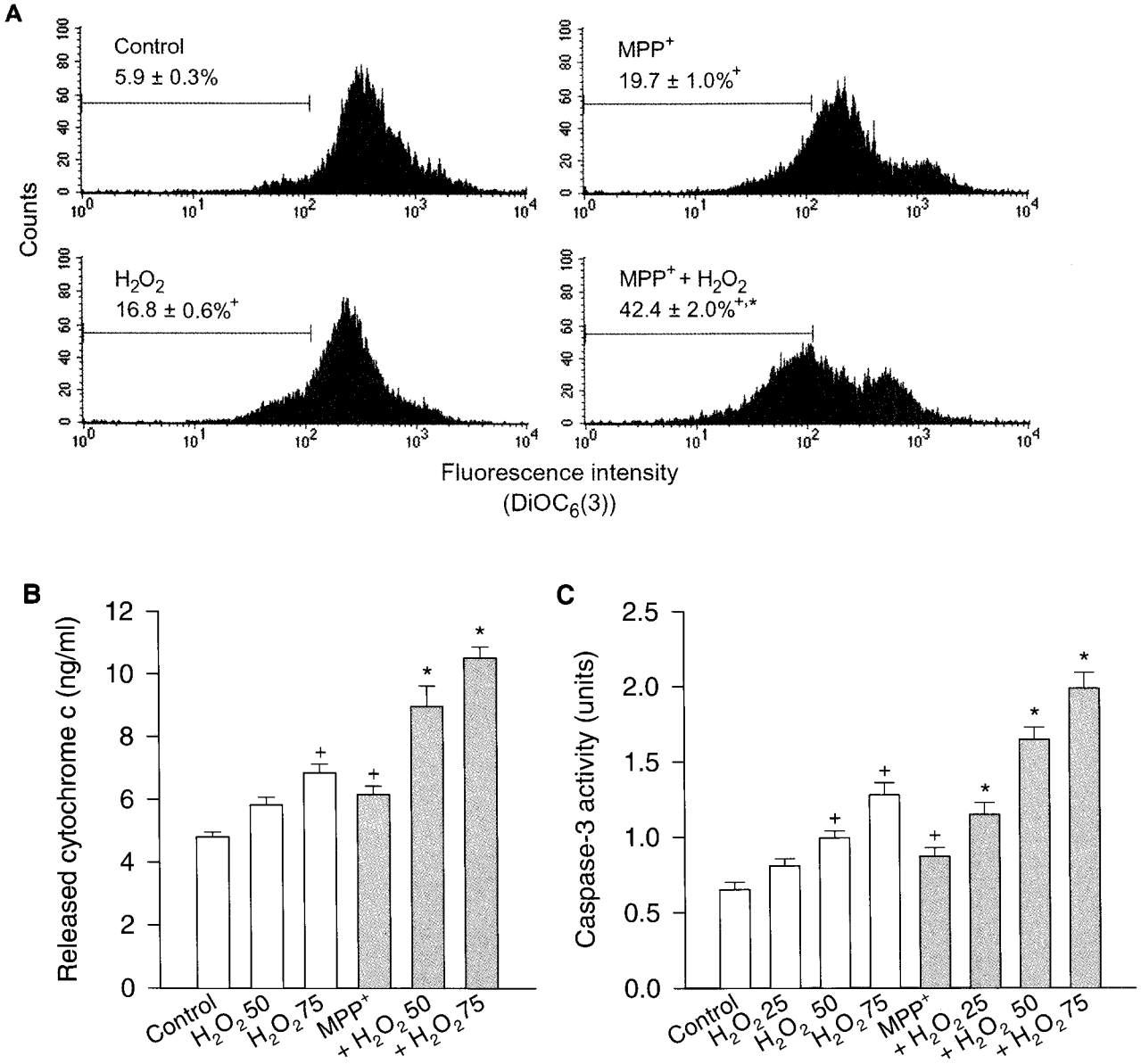

Fig. 4. Increasing effect of H_2O_2 on MPP^+-induced loss of the mitochondrial transmembrane potential, release of cytochrome c and activation of caspase-3. PC12 cells were treated with 130 μM MPP^+ in the presence of 25~75 μM H_2O_2 (75 μM for the mitochondrial membrane potential). Data are expressed as the percentage of cells with depolarized mitochondria for the mitochondrial membrane potential (A), ng/ml for cytochrome c release (B) and units/mg protein for caspase-3 activity (C), and represent means ± SEM (n=3-6). $^+P<0.05$ compared to control; and $^*P<0.05$ compared to MPP^+ alone.

the increase in the cytochrome c release and activation of caspase-3 activity. Treatment with H_2O_2 (25~75 μM) significantly enhanced the MPP^+-induced release of cytochrome c and increase in caspase-3 activity (Fig. 4B, C). The cytochrome c release and caspase-3 activation caused by the co-addition of MPP^+ and H_2O_2 was significantly greater than the sum of that of each compound.

H_2O_2 stimulates MPP^+-induced formation of ROS and depletion of GSH

To determine whether ROS are involved in the combined toxicity of MPP^+ and H_2O_2 in PC12 cells, we investigated the formation of ROS within cells by monitoring a conversion of $DCFH_2$-DA to DCF. We examined the formation of ROS in PC12 cells treated with MPP^+ and H_2O_2. PC12 cells exposed to 130 μM MPP^+ showed about 30% increase in DCF fluorescence, whose response was further enhanced by the addition of 25~75 μM H_2O_2 (Fig. 5A).

Reduction of cellular GSH levels increases the sensitivity of neurons to the toxic effect of neurotoxins and is associated with mitochondrial dysfunction (Chandra et al, 2000). In this study, we investigated the effect of H_2O_2 on the MPP^+-induced decrease in GSH contents. The thiol content in the

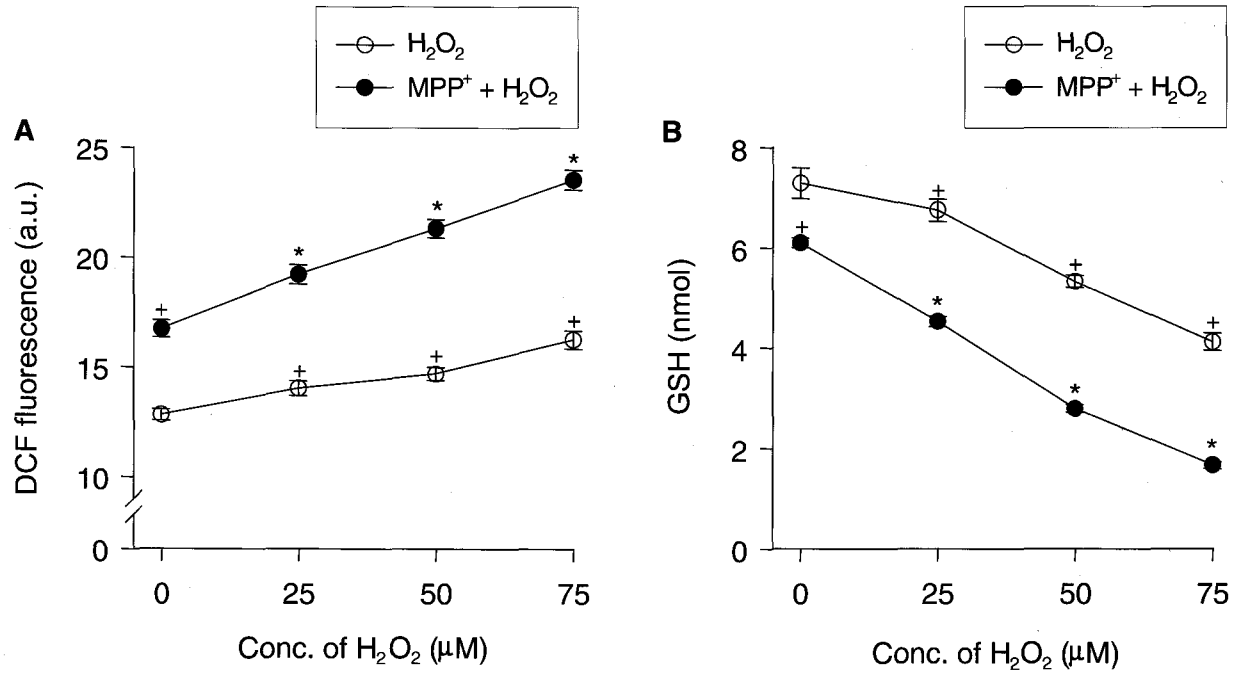

Fig. 5. Effect of H_2O_2 on MPP^+-induced ROS formation and GSH depletion. PC12 cells were treated with 130 μM MPP^+ in the presence of 25~75 μM H_2O_2. Data are expressed as arbitrary units of fluorescence for ROS formation (A) and nmol/μg protein for GSH contents (B) and represent means±SEM (n=6). $^+P<0.05$ compared to control; and $^*P<0.05$ compared to MPP^+ alone.

control PC12 cells was 7.29±0.30 nmol/g protein. Treatment with 130 μM MPP^+ for 24 h depleted GSH contents by 16%. H_2O_2 (25~75 μM) significantly enhanced the MPP^+-induced depletion of GSH (Fig. 5B). The GSH depletion due to co-addition of MPP^+ and H_2O_2 was greater than the sum of that of each compound.

DISCUSSION

Although rat PC12 cells are not brain dopaminergic neurons, these cells are able to produce dopamine and express dopamine transporter (Kadota et al, 1996). Upon nerve growth factor stimulation, PC12 cells not only display abundant neuritic growth, but also adopt a neurochemical dopaminergic phenotype. On the basis of the character of PC12 cells, we assessed the cytotoxicity of MPP^+ and H_2O_2 against dopaminergic neurons using the PC12 cells differentiated with nerve growth factor. The cytotoxic effect of MPP^+ and H_2O_2 on cell viability in differentiated PC12 cells was demonstrated by using MTT assay and by observing nuclear morphological changes with Hoechst 33258 stain, which indicated necrotic and apoptotic cell death. Opening of the mitochondrial permeability transition pore causes a release of cytochrome c from mitochondria into the cytosol, leading to activation of caspase-3 activation that is involved in apoptotic cell death (Crompton, 1999). In this study, the MPP^+- or H_2O_2-induced apoptotic cell death in differentiated PC12 cells was demonstrated by the condensation and fragmentation of nuclei and by changes in the mitochondrial membrane permeability, leading to the cytochrome c release and caspase-3 activation.

It has been shown that the H_2O_2 treatment elicits the alteration of mitochondrial structure and function, leading to the formation of ROS and nitrogen species, and the activation of the mitochondrial permeability transition (Chandra et al, 1999; Hong & Liu, 2004). The H_2O_2-induced apoptosis in neuronal cells seems to be mediated by loss of the mitochondrial transmembrane potential, results in the release of mitochondrial cytochrome c and subsequent activation of caspase-3 (Cardoso et al, 2004; Mronga et al, 2004). In agreement with these reports, in this study PC12 cells exposed to 25~75 μM of H_2O_2 exhibited mitochondrial damage, leading to the activation of caspase-3. The ROS formation and GSH depletion in PC12 cells suggests that the H_2O_2-induced mitochondrial dysfunction and cell death may be caused by oxidative stress. The inhibition of mitochondrial respiratory chain caused by oxidants produces ROS and nitrogen species (Brown, 1999; Fleury et al, 2002). During the apoptotic process, drops in GSH levels and concomitant increase in ROS are detected (Tan et al, 1998; Chandra et al, 2000). The mitochondrial membrane permeability transition could induce formation of ROS and nitrogen species by inhibition of respiratory chain (Fleury et al, 2002; Polster & Fiskum, 2004). The inhibitory effect of antioxidants, including *N*-acetylcysteine and Mn-TBAP suggests that treatment either with MPP^+ and H_2O_2 or with H_2O_2 alone induces the formation of ROS and nitrogen species in PC12 cells, whose response causes mitochondrial dysfunction.

The concomitant addition of H_2O_2 and either tumor necrosis factor or ethanol reveals a synergistic effect on cell death in PC12 cells (Trembovler et al, 2003; Lee et al, 2005). MPP^+ is demonstrated to stimulate the displacement of dopamine from vesicular storage sites to the cytoplasm, which further induces oxidation of dopamine (Lotharius & O'Malley, 2000). The oxidation of dopamine liberates ROS and toxic quinines. This finding indicates

that cytotoxicity of MPP^+ is mediated by dopamine oxidation. However, it has been suggested that the cell death due to co-addition of dopamine and MPP^+ is not mediated by ROS (Lee et al, 2003). The aim of this study was to explore whether the MPP^+-induced changes in the mitochondrial membrane permeability are modulated by H_2O_2. In this study, H_2O_2 exhibited a stimulatory effect on the cytotoxicity of MPP^+. The present results suggest that H_2O_2 enhances the MPP^+-induced cell death in differentiated PC12 cells by promoting the loss of mitochondrial transmembrane potential, cytochrome c release and subsequent caspase-3 activation. Cell permeable H_2O_2 shows a depletion of mitochondrial GSH (Chandra et al, 2000). Depletion of mitochondrial GSH increases the formation of ROS, and the oxidation and depletion of GSH induce formation of the mitochondrial membrane permeability transition (Constantini et al, 1996; Chandra et al, 2000). In the present study, the stimulatory effect of H_2O_2 on the MPP^+-induced cell death approximately correlated with the effect on GSH depletion. Therefore, the promoting effect of H_2O_2 on the MPP^+-induced changes in the mitochondrial membrane permeability may be accomplished by enhancement of ROS formation and depletion of cellular GSH.

Overall, the results show that treatment with H_2O_2 promotes the cytotoxicity of MPP^+ against PC12 cells. H_2O_2 may enhance the MPP^+-induced viability loss in PC12 cells by promoting changes in the mitochondrial membrane permeability, leading to the release of cytochrome c, which is associated with the increased formation of ROS and depletion of GSH. The findings suggest that H_2O_2 as a promoting agent for the formation of mitochondrial permeability transition may enhance the neuronal cell injury caused by neurotoxins.

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