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Synergistic effects of hydrogen peroxide and ethanol on cell viability loss in PC12 cells by increase in mitochondrial permeability transition

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Abstract

The promoting effect of ethanol against the cytotoxicity of hydrogen peroxide (H_2O_2) in differentiated PC12 cells was assessed by measuring the effect on the mitochondrial membrane permeability. Treatment of PC12 cells with H_2O_2 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. In PC12 cells and dopaminergic neuroblastoma SH-SY5Y cells, the promoting effect of ethanol on the H_2O_2-induced cell death was increased with exposure time. Ethanol promoted the nuclear damage, change in the mitochondrial membrane permeability, ROS formation and decrease in GSH contents due to H_2O_2 in PC12 cells. Catalase, carboxy-PTIO, Mn-TBAP, *N*-acetylcysteine, cyclosporin A and trifluoperazine inhibited the H_2O_2 and ethanol-induced mitochondrial dysfunction and cell injury. The results show that the ethanol treatment promotes the cytotoxicity of H_2O_2 against PC12 cells. Ethanol may enhance the H_2O_2-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 ethanol as a promoting agent for the formation of mitochondrial permeability transition may enhance the neuronal cell injury caused by oxidants.

Keywords: Hydrogen peroxide; Ethanol; PC12 cells; Mitochondrial membrane permeability; Cell injury

1. 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 [1,2]. 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 MPP^+ [3–5]. 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 [6]. The co-addition of dopamine and MPP^+ shows an enhancing effect on the mitochondrial membrane permeability change and cell death [7]. 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 [5,8].

Hydrogen peroxide, one of the products of dopamine oxidation, diffuses partly into the mitochondrial matrix and oxidizes GSH to glutathione disulphide, which is thought to induce the alteration of cellular functions, including suppression of the thiol-dependent electron transport [9]. Inhibition of the mitochondrial respiratory chain enhances

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

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superoxide formation that can initiate apoptotic cell death through a decrease in the mitochondrial membrane potential [10]. It has been shown that the H_2O_2-induced cell death is mediated by mitochondrial damage and cytochrome *c*, which accompanied by the activation of caspase-3 and 9 [11], by upregulation of the Fas receptor/ligand system [12] and by disruption of the intracellular Ca^{2+} homeostasis [13].

Ethanol has been demonstrated to induce apoptotic cell death in neuronal cells [14,15]. The ethanol-induced cell death is mediated by mitochondrial dysfunction and activation of the signaling pathway implicated in cell death [16,17]. Ethanol exposure causes the reduction of endogenous antioxidant levels and the formation of ROS, the depletion of GSH and the DNA fragmentation [18,19]. Ethanol shows a toxic effect against rat cerebral cortex and cerebellum by inducing mitochondrial dysfunction, leading to the release of cytochrome *c* and activation of caspases [20]. In ethanol-exposed cells, the induction of the mitochondrial permeability transition may be increased by various agents, including the proapoptotic protein Bax [21]. Ethanol enhances the cytotoxicity of tumor necrosis factor-α by potentiating induction of the mitochondrial membrane permeability transition [19]. The ethanol-induced cell death in astrocytes seems to be mediated by ceramide signaling pathways triggering apoptosis and the addition of C2-ceramide is found to potentiate the cytotoxic effect of ethanol [17].

The dopamine oxidation products and MPP^+ are suggested to reveal oxidative forms of neuronal cell injury through the induction of the membrane permeability transition. Ethanol may promote the formation of the mitochondrial permeability transition by the proapoptotic protein. However, the effect of ethanol on the oxidant-mediated toxicity in neuronal cells has not been elucidated. The aim of the present study was to investigate whether ethanol acts as a promoting agent against oxidative neuronal cell injury. We examined the stimulating effect of ethanol against the cytotoxicity of H_2O_2 in relation to change in the mitochondrial membrane permeability using differentiated PC12 cells and human dopaminergic neuroblastoma SH-SY5Y cells.

2. Materials and methods

2.1. Materials

TiterTACS™ colorimetric apoptosis detection kit was purchased from Trevigen Inc., Quantikine® M rat/mouse cytochrome *c* assay kit was from R&D systems, ApoAlert™ CPP32/caspase-3 assay kit was from CLONTECH Laboratories Inc. and Mn-TBAP was from OXIS International Inc. Catalase (from bovine liver; 10,000–25,000 U/mg protein), carboxy-PTIO, MTT, $DiOC_6(3)$, $DCFH_2$-DA, DTNB, PMSF and other chemicals were purchased from Sigma–Aldrich Inc. Protein concentration was determined by the method of Bradford according to the instructions of Bio-Rad Laboratories.

2.2. Cell culture

Rat PC12 cells (adrenal gland; pheochromocytoma) and human dopaminergic neuroblastoma SH-SY5Y cells 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 (FBS), 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 [22]. Cells were washed with RPMI medium containing 1% FBS 24 h before experiments and replated onto the 96- and 24-well plates. SH-SY5Y cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin as described in the previous report [23]. The experiments using SH-SY5Y cells were performed in DMEM medium.

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 [24]. PC12 cells or SH-SY5Y cells (4×10^4 cells/200 μl) were treated with H_2O_2 in the presence of ethanol 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.). Cell viability was expressed as a percentage of the value in control cultures.

2.4. Morphological observation of nuclear change

PC12 cells (1×10^6 cells/ml) were treated with H_2O_2 for 24 h at 37 °C and the nuclear morphological change was assessed using the Hoechst dye 33258 [25]. 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).

2.5. 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 H_2O_2 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.

2.6. Flow cytometric measurement of mitochondrial transmembrane potential

Changes in the mitochondrial transmembrane potential during the H_2O_2-induced apoptosis in PC12 cells were quantified by flow cytometry with the cationic lipophilic dye $DiOC_6(3)$ [26]. 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 $412 \times g$ 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) with argon laser excitation at 501 nm was used to assess 10,000 cells from each sample.

2.7. 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 $412 \times g$ 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 $100{,}000 \times g$ 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 nanograms per millilitre by reference to the standard curve.

2.8. Measurement of caspase-3 activity

PC12 cells (2×10^6 cells/ml) were treated with H_2O_2 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.

2.9. 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 [27]. PC12 cells (4×10^4 cells/200 μl) were treated with H_2O_2 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 PBS. The cell suspensions were centrifuged at $412 \times g$ 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).

2.10. Measurement of total glutathione

The total glutathione (reduced form GSH + oxidized form GSSG) was determined using glutathione reductase [28]. PC12 cells (4×10^4 cells/200 μl) were treated with H_2O_2 for 24 h at 37 °C, centrifuged at $412 \times g$ 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 and 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.

2.11. Statistical analysis

Data are expressed as 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 comparisons. A probability less than 0.05 was considered to be statistically significant. Depiction and expression of the data in the figures were based on the previous reports.

3. Results

3.1. Ethanol enhances H_2O_2-induced cell death and nuclear damage

The effect of various concentrations of ethanol on the cytotoxicity of H_2O_2 was assessed in PC12 cells that are

differentiated by nerve growth factor. After addition of 75 μM H_2O_2, the incidence of cell death at a 24-h incubation was about 44% in PC12 cells. The concentrations of ethanol used in this study were based on the previous reports [14,15,17]. Pre-treatment with ethanol (40–200 mM) significantly enhanced the 75 μM H_2O_2-induced cell death in PC12 cells in a dose-dependent manner. The cytotoxic effect of H_2O_2 plus ethanol was apparently greater than the sum of that of each compound (Fig. 1A). When PC12 cells were treated with 75 μM H_2O_2 in the presence of 60 mM ethanol, cell death was increased with exposure time (Fig. 1B). The present study examined whether the synergistic effect of ethanol on the cytotoxicity of H_2O_2 is also detected in other dopaminergic cell line. After addition of 75 μM H_2O_2 to SH-SY5Y cells, the incidence of cell death at 24 h was about 38%. Similar to PC12 cells, when SH-SY5Y cells were exposed to 75 μM H_2O_2 plus 60 mM ethanol, cell death was increased with time (Fig. 1C). Both cell lines exposed to 60 mM ethanol revealed 10–14% cell viability loss.

We examined whether the toxic effect of H_2O_2 and ethanol against PC12 cells is mediated by the actions of ROS and nitrogen species. Treatment with 10 μg/ml catalase (a scavenger of hydrogen peroxide), 25 μM carboxy-PTIO (a scavenger of nitric oxide), 30 μM Mn-TBAP (a scavenger of peroxynitrite and cell-permeable metalloporphyrin that mimics superoxide dismutase), 1 mM thiol compound *N*-acetylcysteine and inhibitors of the mitochondrial permeability transition (0.5 μM of cyclosporin A and trifluoperazine) reduced cell death caused by either H_2O_2 alone or H_2O_2 plus ethanol (Fig. 2A and B).

To assess apoptotic cell death due to H_2O_2 and clarify the stimulatory effect of ethanol against the cytotoxicity of H_2O_2, we investigated the effect of ethanol on the nuclear morphological changes observed in the H_2O_2-treated cells. 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 75 μM H_2O_2 and 60 mM ethanol (Fig. 3A). *N*-Acetylcysteine (1 mM) depressed the H_2O_2 plus ethanol-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 75 μM H_2O_2 in the presence or absence of ethanol. Control cells showed absorbance of 0.210 ± 0.009 (mean ± S.E.M. of six experiments), whilst exposure to 75 μM H_2O_2 for 24 h increased the absorbance about 2.2-fold (Fig. 3B). Despite the damaging effect, 60–100 mM ethanol significantly

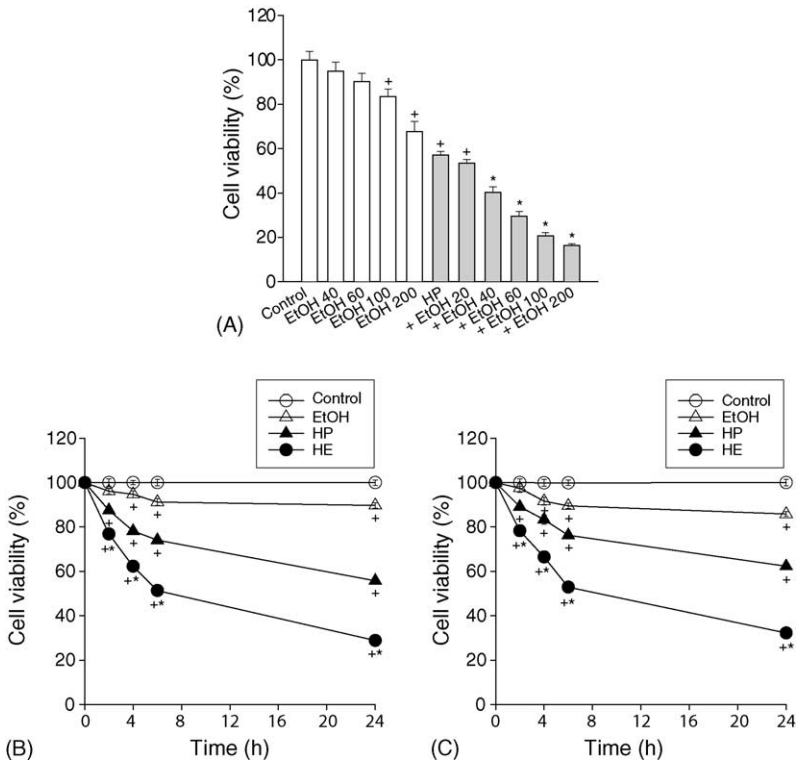

Fig. 1. Effect of ethanol on H_2O_2-induced cell death. In experiment A, PC12 cells were pre-treated with ethanol (20–200 mM) for 30 min, added 75 μM H_2O_2 to the mixture and cell viability at a 24-h incubation was determined. In experiment B, PC12 cells were pre-treated with 60 mM ethanol, added 75 μM H_2O_2 to the mixture and at the designated times cell death was determined. In experiment C, as in experiment B the SH-SY5Y cells were treated with 60 mM and 75 μM H_2O_2. Data represent mean ± S.E.M. of six replicate values in two separate experiments; $^{+}p < 0.05$ compared to control (percentage of control) and $^{*}p < 0.05$ compared to H_2O_2 alone. H_2O_2 was expressed as HP, ethanol as EtOH and H_2O_2 plus ethanol as HE.

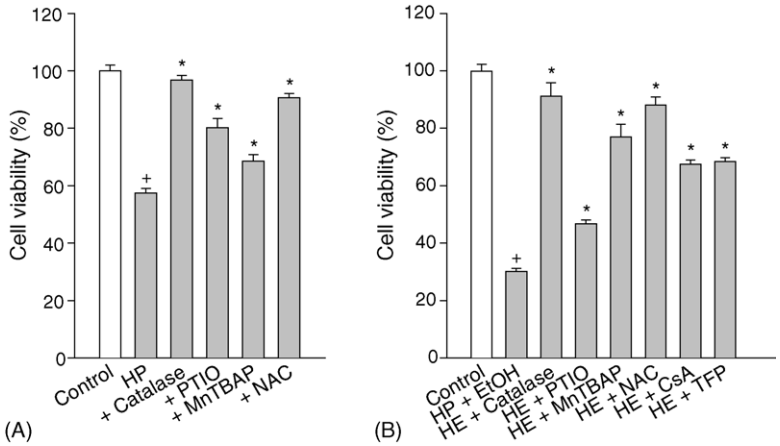

Fig. 2. Inhibition of the H_2O_2 and ethanol-induced cell death by antioxidants. In experiment A, cells were pre-treated with the scavengers [10 μg/ml catalase, 25 μM carboxy-PTIO (PTIO), 30 μM Mn-TBAP (MnTBAP) and 1 mM *N*-acetylcysteine (NAC)], added 75 μM H_2O_2 to the mixture and cell viability at 24 h was determined. In experiment B, cells were pre-treated with 60 mM ethanol in the presence of compounds [scavengers, 0.5 μM cyclosporin A (CsA) or 0.5 μM trifluoperazine (TFP)] and then added 75 μM H_2O_2 to the mixture. Data represent mean ± S.E.M. of six replicate values in two separate experiments. In A, $^{+}p < 0.05$ compared to control and $^{*}p < 0.05$ compared to H_2O_2 alone. In B, $^{+}p < 0.05$ compared to control and $^{*}p < 0.05$ compared to H_2O_2 plus ethanol. H_2O_2 was expressed as HP, ethanol as EtOH and H_2O_2 plus ethanol as HE.

enhanced the fragmentation of DNA due to H_2O_2. The increase in absorbance due to co-addition of H_2O_2 and ethanol was greater than the sum of that of each compound.

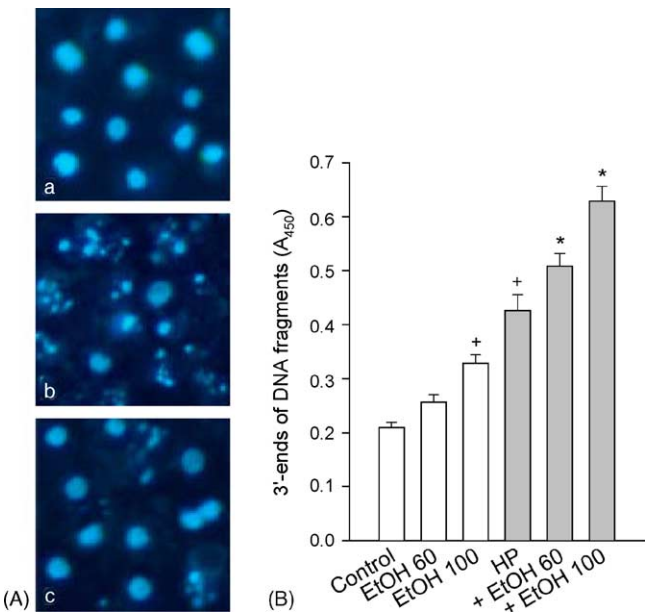

Fig. 3. Enhancement of H_2O_2-induced nuclear damage by ethanol. PC12 cells were pre-treated with 60–100 mM ethanol and then added 75 μM H_2O_2 to the mixture. In experiment A, cells were observed by fluorescence microscopy after nuclei staining with Hoechst 33258. Figure represents microscopic morphology of the control cells (a), cells treated with H_2O_2 plus 60 mM ethanol (b) and cells treated with H_2O_2 and ethanol in the presence of 1 mM *N*-acetylcysteine (c); (a)–(c) are representatives of four different experiments. In experiment B, the 3′-ends of DNA fragments were detected as described in Section 2. Data are expressed as absorbance and represent mean ± S.E.M. of six replicate values in two separate experiments; $^{+}p < 0.05$ compared to control and $^{*}p < 0.05$ compared to H_2O_2 alone. H_2O_2 was expressed as HP and ethanol as EtOH.

3.2. Ethanol enhanced H_2O_2-induced changes in mitochondrial membrane permeability

We assessed the cytotoxic effect of H_2O_2 by investigating its effect on the mitochondrial membrane permeability. Change in the mitochondrial transmembrane potential in PC12 cells treated with H_2O_2 was quantified by flow cytometry with the dye $DiOC_6(3)$. Exposure of PC12 cells to 75 μM H_2O_2 for 24 h increased the percentage of cells with depolarized mitochondria (characterized by low values of the transmembrane potential). Ethanol (60–100 mM) significantly enhanced the H_2O_2-induced increase in cells with depolarized mitochondria (Fig. 4A).

The H_2O_2-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 75 μM H_2O_2 showed a significant increase in the cytochrome *c* release and activation of caspase-3 activity. Ethanol (60–100 mM) significantly enhanced the H_2O_2-induced release of cytochrome *c* and increase in caspase-3 activity (Fig. 4B and C). The cytochrome *c* release and caspase-3 activation caused by the co-addition of H_2O_2 and ethanol was apparently greater than the sum of that of each compound. Addition of 10 μg/ml catalase, 25 μM carboxy-PTIO, 30 μM Mn-TBAP and 1 mM *N*-acetylcysteine significantly attenuated the H_2O_2 and ethanol-induced cytochrome *c* release and caspase-3 activation (Fig. 5A and B).

3.3. Ethanol stimulated H_2O_2-induced formation of ROS and depletion of GSH

To determine whether ROS are involved in the H_2O_2 plus ethanol-induced cell death in PC12 cells, we inves-

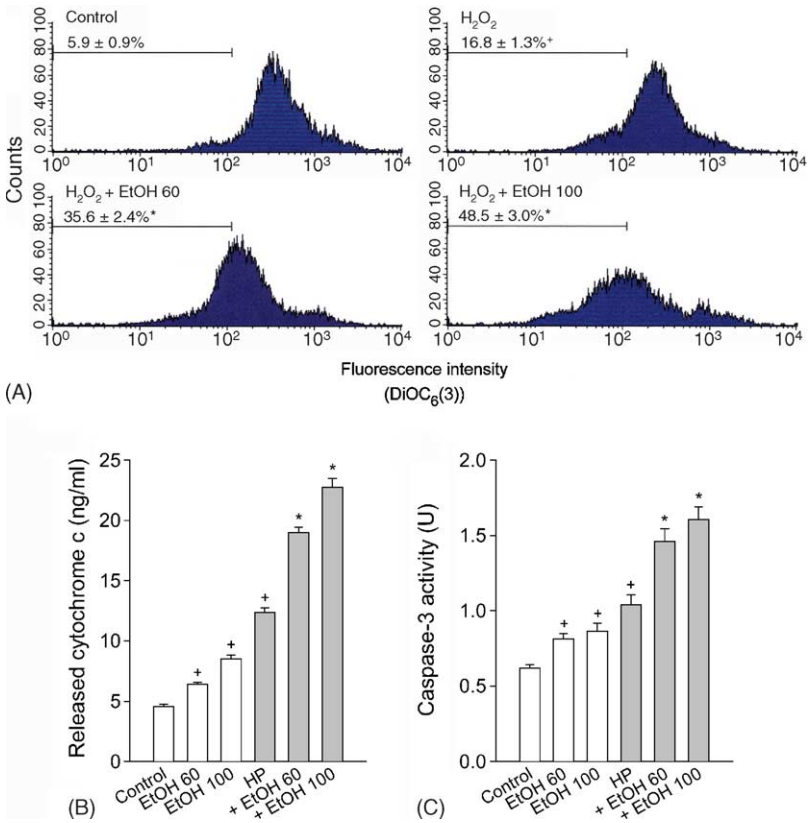

Fig. 4. Effect of ethanol on loss of the mitochondrial transmembrane potential, release of cytochrome *c* and activation of caspase-3 due to H_2O_2. PC12 cells were pre-treated with 60–100 mM ethanol and then added 75 μM H_2O_2 to the mixture. Data are expressed as the percentage of cells with depolarized mitochondria for the mitochondrial membrane potential (A), ng/ml for cytochrome *c* release (B) and U/μg protein for caspase-3 activity (C) and represent mean ± S.E.M. of three to six replicate values in two to three separate experiments; $^{+}p < 0.05$ compared to control and $^{*}p < 0.05$ compared to H_2O_2 alone. H_2O_2 was expressed as HP and ethanol as EtOH.

tigated 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 75 μM H_2O_2 and ethanol. PC12 cells treated with 75 μM H_2O_2 showed a significant increase in DCF fluorescence, a response that was further increased by the addition of 60–100 mM ethanol (Fig. 6A). Catalase (10 μg/ml), 25 μM carboxy-PTIO, 30 μM Mn-TBAP and 1 mM

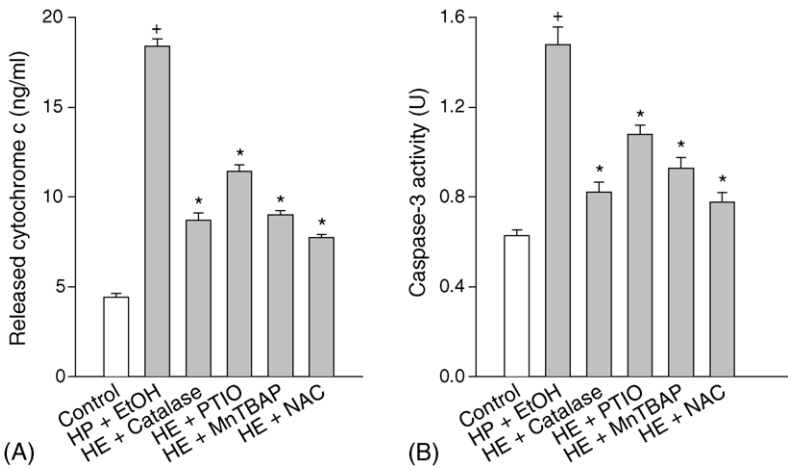

Fig. 5. Inhibition of the H_2O_2 and ethanol-induced cytochrome *c* release by antioxidants. PC12 cells were pre-treated with 60 mM ethanol in the presence of the scavengers [10 μg/ml catalase, 25 μM carboxy-PTIO (PTIO), 30 μM Mn-TBAP (MnTBAP) and 1 mM *N*-acetylcysteine (NAC)] and then added 75 μM H_2O_2 to the mixture. After a 24-h incubation, the cytochrome *c* released (A) and caspase-3 activity (B) were determined. Data represent mean ± S.E.M. of six replicate values in two separate experiments; $^{+}p < 0.05$ compared to control and $^{*}p < 0.05$ compared to H_2O_2 alone. H_2O_2 was expressed as HP, ethanol as EtOH, and H_2O_2 plus ethanol as HE.

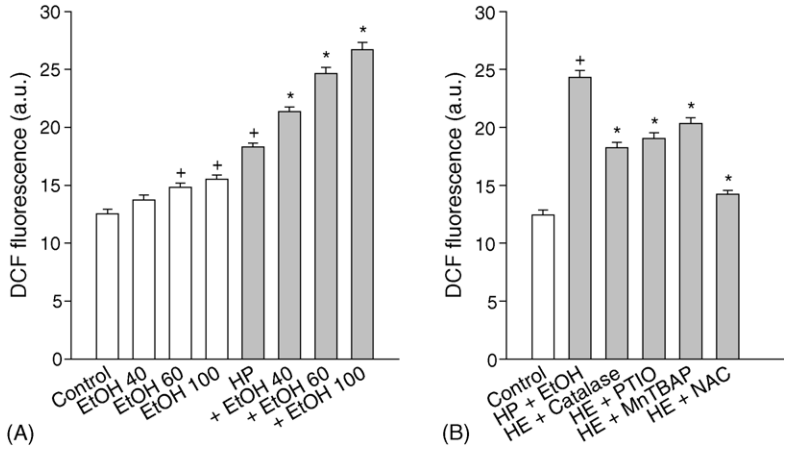

Fig. 6. Effect of ethanol on H_2O_2-induced ROS formation. In experiment A, PC12 cells were pre-treated with 40–100 mM ethanol and then added 75 μM H_2O_2 to the mixture. In experiment B, cells were pre-treated with 60 mM ethanol in the presence of the scavengers [10 μg/ml catalase, 25 μM carboxy-PTIO (PTIO), 30 μM Mn-TBAP (MnTBAP) and 1 mM *N*-acetylcysteine (NAC)] and added 75 μM H_2O_2 to the mixture. Data are expressed as arbitrary units of fluorescence and represent mean ± S.E.M. of six replicate values in two separate experiments. In A, $^{+}p < 0.05$ compared to control and $^{*}p < 0.05$ compared to H_2O_2 alone. In B, $^{+}p < 0.05$ compared to control and $^{*}p < 0.05$ compared to H_2O_2 plus ethanol. H_2O_2 was expressed as HP, ethanol as EtOH, and H_2O_2 plus ethanol as HE.

N-acetylcysteine inhibited the increase in DCF fluorescence due to H_2O_2 plus ethanol (Fig. 6B).

Reduction of cellular GSH levels increases the sensitivity of neurons to the toxic effect of neurotoxins and is associated with mitochondrial dysfunction [29]. In this study, we investigated the effect of ethanol on the H_2O_2-induced decrease in GSH contents. The thiol content in the control PC12 cells was 3.61 ± 0.10 nmol/μg protein. Treatment with 75 μM H_2O_2 for 24 h depleted GSH contents by 45%. Ethanol (60–100 mM) significantly enhanced the H_2O_2-induced depletion of GSH (Fig. 7A). The GSH depletion due to co-addition of H_2O_2 and ethanol was greater than the sum of that of each compound. Treatment with 10 μg/ml catalase, 25 μM carboxy-PTIO and 30 μM Mn-TBAP significantly inhibited the H_2O_2 and ethanol-induced decrease in the GSH contents (Fig. 7B).

4. Discussion

The pathologic feature in Parkinson's disease reveals the striking degenerative loss of dopaminergic neurons in the nigrostriatal system. Although rat PC12 cells are not brain dopaminergic neurons, these cells are able to produce dopamine and express dopamine transporter [30]. Upon nerve growth factor stimulation, PC12 cells not only display abundant neuritic growth, but also adopt a neurochemical dopaminergic phenotype. SH-SY5Y cell is a human neuroblastoma cell line and has catecholaminergic properties [31]. These cells are very sensitive to oxidative stress, such as oxidative attack of 6-hydroxydopamine [32]. Therefore, this cell line is frequently used to assess the neuronal survival of dopaminergic neurons [23,33]. On the basis of the character of both cell lines, we assessed the

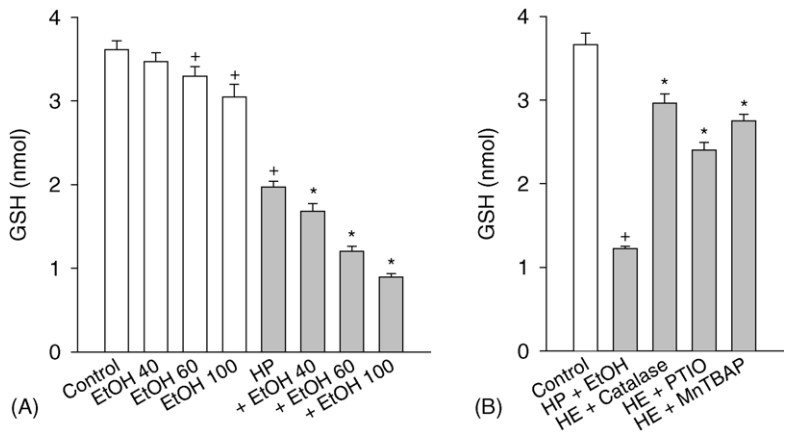

Fig. 7. Enhancement of H_2O_2-induced decrease in the GSH contents by ethanol. In experiment A, PC12 cells were pre-treated with 40–100 mM ethanol and then added 75 μM H_2O_2 to the mixture. In experiment B, cells were pre-treated with 60 mM ethanol in the presence of the scavengers [10 μg/ml catalase, 25 μM carboxy-PTIO (PTIO) and 30 μM Mn-TBAP (MnTBAP)] and added 75 μM H_2O_2 to the mixture. Data are expressed as nmol of GSH/μg protein and represent mean ± S.E.M. of six replicate values in two separate experiments. $^{+}p < 0.05$ compared to control and $^{*}p < 0.05$ compared to H_2O_2 alone. H_2O_2 was expressed as HP, ethanol as EtOH, and H_2O_2 plus ethanol as HE.

cytotoxicity of H_2O_2 against dopaminergic neurons using the PC12 cells differentiated with nerve growth factor and the SH-SY5Y cells. A significant cytotoxic effect of 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. In PC12 and SH-SY5Y cells, the incidences of cell death due to H_2O_2 or ethanol were increased with exposure time. The cytotoxic effect of H_2O_2 plus ethanol was apparently greater than the sum of that of each compound. The results suggest that ethanol shows a similar promoting effect on the toxic effect of H_2O_2 in both neuronal cells, which have dopaminergic cell line character. 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 [2]. In this study, the 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 chronic ethanol 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 [16,34]. The ethanol-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 [15,19]. In agreement with these reports, in this study PC12 cells exposed to high concentrations of ethanol exhibited a mitochondrial damage that induces the activation of caspase-3. The formation of ROS in PC12 cells suggests that the ethanol-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 [35,36]. Cells exposed to H_2O_2 reveal the formation of ROS, a response that is attenuated by antioxidants [29,37]. The inhibitory effect of antioxidants, including Mn-TBAP and *N*-acetylcysteine also suggests that H_2O_2 induces the formation of ROS and nitrogen species in PC12 cells and the mitochondrial dysfunction due to H_2O_2 is mediated by oxidative stress.

Mitochondria isolated from ethanol-fed rats are more sensitive to induction of the mitochondrial permeability transition by the agents, including atractyloside (an inducer of the mitochondrial permeability transition) and Bax, than their pair matched controls [21]. Ethanol enhances the tumor necrosis factor-α-induced cell death by promoting the mitochondrial depolarization, release of cytochrome *c* and activation of caspase-3 [19]. The aim of this study was to explore whether the H_2O_2-induced changes in the mitochondrial membrane permeability are modulated by ethanol. In this study, ethanol exhibited a stimulatory effect on the cytotoxicity of H_2O_2. The present results suggest that ethanol enhances the H_2O_2-induced cell death in differentiated PC12 cells by promoting the loss of mitochondrial transmembrane potential, cytochrome *c* release and subsequent caspase-3 activation. The inhibitory effect of cyclosporin A and trifluoperazine also suggests that ethanol may enhance the H_2O_2-induced cell death by promoting induction of the mitochondrial permeability transition. The inhibitory effect of antioxidants, such as Mn-TBAP and *N*-acetylcysteine, also indicates that mitochondrial dysfunction leading to caspase-3 activation is caused by oxidative stress. Ethanol feeding shows a depletion of liver mitochondrial GSH [38]. Depletion of mitochondrial GSH is demonstrated to increase the formation of ROS, and the oxidation and depletion of GSH induce formation of the mitochondrial membrane permeability transition in rats [29,39]. In the present study, the stimulatory effect of ethanol on the H_2O_2-induced cell death approximately correlated with the effect on GSH depletion. Therefore, the promoting effect of ethanol on the H_2O_2-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 ethanol promotes the cytotoxicity of H_2O_2 against PC12 cells. Ethanol may enhance the H_2O_2-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 ethanol as a promoting agent for the formation of mitochondrial permeability transition may enhance the neuronal cell injury caused by oxidants. Although the concentrations of ethanol used in this study are high, the results postulate that in a long-term exposure state low concentration of ethanol seems to enhance the oxidative neuronal cell injury in vivo.

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