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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.
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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

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