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Effects of (1R,9S)-β-Hydrastine hydrochloride on L-DOPA-Induced Cytotoxicity in PC12 cells

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Abstract – Previously, (1R,9S)-β-hydrastine hydrochloride has been found to lower dopamine content in PC12 cells (Kim *et al.*, 20001). In this study, the effects of (1R,9S)-β-hydrastine hydrochloride on L-DOPA-induced cytotoxicity in PC12 cells were investigated. Treatment with (1R,9S)-β-hydrastine hydrochloride at concentrations higher than 500 μM caused cytotoxicity in PC12 cells. In addition, (1R,9S)-β-hydrastine hydrochloride at non-cytotoxic or cytotoxic concentrations significantly enhanced L-DOPA-induced cytotoxicity (L-DOPA concentration, 50 μM). Treatment of PC12 cells with 750 μM 1R,9S)-β-hydrastine hydrochloride and 50 μM L-DOPA, alone or in combination, also induced cell death via a mechanism which exhibited morphological and biochemical characteristics of apoptosis, including chromatin condensation and membrane blebbing. Exposure of PC12 cells to (1R,9S)-β-hydrastine hydrochloride, L-DOPA and (1R,9S)-β-hydrastine hydrochloride plus L-DOPA for 48 h resulted in a marked increase in the cell loss and percentage of apoptotic cells compared with exposure for 24 h. These data indicate that (1R,9S)-β-hydrastine hydrochloride at higher concentration ranges aggravates L-DOPA-induced neurotoxicity cytotoxicity in PC12 cells. Therefore, it is proposed that the long-term L-DOPA therapeutic patients with (1R,9S)-β-hydrastine hydrochloride could be checked for the adverse symptoms.
Keywords – (1R,9S)-β-Hydrastine hydrochloride, L-DOPA, Cytotoxicity, PC12 cells, Apoptosis

Introduction

Hydrastine derivatives are composed of a phthalide and an isoquinoline alkaloid, have been isolated from the roots of *Hydrastis canadensis* L. (Ranunculaceae) and *Berberis laurina* Billb. (Berberidaceae) (Stanek and Manske, 1968; Tang and Eisenbrand, 1992). (1R,9S)-β-Hydrastine has been found to competitively inhibit bovine adrenal tyrosine hydroxlase (EC 1.14.16.2; TH) with L-tyrosine as a substrate (Lee *et al.*, 1997). Recently, (1R,9S)-β-hydrastine hydrochloride at lower concentration ranges of 10-50 μM inhibits dopamine biosynthesis, in part, through the inhibition of TH activity in PC12 cells (Kim *et al.*, 2001).

Isoquinoline derivatives (IQs) structurally related to the selective dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+), have emerged as candidate endogenous neurotoxins causing nigral cell death in Parkinson's disease (McNaught *et al.*, 1998; Ohta, 2000). IQs are widely distributed in the environment, being present in many plants, foodstuffs (e.g. cocoa, milk, and bananas) and alcoholic beverages (Makino *et al.*, 1988), and readily cross the blood-brain barrier (Kikuchi *et al.*, 1991). Among IQs, tetrahydroisoquinoline (TIQ), 1-benzyl-TIQ and (R)-1,2-dimethyl-5,6-dihydroxy-TIQ [(R)-N-methylsalsolinol] have the most potent neurotoxicity (Tasaki *et al.*, 1991; Kotake *et al.*, 1995; Maruyama *et al.*, 1996). Hydrastine derivatives have the same TIQ configuration as the TIQ. Therefore, it is conceivable that hydrastine derivatives might cause neurodegeneration.

L-3,4-Dihydroxyphenylalanine (levodopa or L-DOPA), which is the amino acid precusor of dopamine, is the most frequently prescribed drug for controlling the symptoms of Parkinson's disease (Marsden, 1994). However, some reports have suggested that L-DOPA may accelerate the deterioration of Parkinsonian patients and that L-DOPA toxicity occurs in damaged dopamine neurons in vivo (Perry *et al.*, 1984). It is also reported that L-DOPA produces cytotoxicity generating reactive oxygen species leading to apoptosis due to autoxidation and enzymatic oxidation (Sandstrom *et al.*, 1994).

The pheochromocytoma, PC12, cell lines are originally

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characterized from a catecholamine-secreting adrenal chromaffin tumor in rats (Greene and Tischler, 1976) and have been widely used to investigate neuronal differentiation, signal transduction and neuronal cell death (Itano *et al.*, 1994).

In this study, therefore, the effects of with (1R,9S)-β-hydrastine hydrochloride on L-DOPA-induced cytotoxicity in PC12 cells were investigated.

Materials and Methods

Chemicals – (1R,9S)-β-Hydrastine hydrochloride, L-DOPA, RNase A, propidium iodide (PI), ethylenediaminetetraacetic acid (EDTA), poly-L-lysine and 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from the Sigma (St. Louis, MO, USA). The in situ cell death detection kit (terminal deoxynucleotidyltransferase dUTP nick-end labeling: TUNEL) was supplied from the Boehringer Mannheim (Mannheim, Germany). All sera, antibiotics and RPMI 1640 for cell culture were obtained from the Gibco (Grand Island, NY, USA). All other chemicals were of reagent grade.

Cell culture – PC12 cells were routinely maintained in RPMI 1640 medium supplemented with 10% heat-inactivated donor horse serum and 5% heat-inactivated fetal calf serum plus 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C.

Assessment of cell viability – Cell viability was determined by the conventional MTT assay with slight modification (Mosman, 1983). PC12 cells were diluted to 1×10^5 cells/ml and plated at 100 μl per well in a 96 well microplate. PC12 cells were treated with various concentrations of (1R,9S)-β-hydrastine hydrochloride (50-750 μM) and L-DOPA (20-100 μM), alone or in combination, for 24 h or 48 h. The MTT solution (final concentration 1 mg/ml) was added to the cells and the cultures allowed to incubate at 37°C for 3-4 h. After the supernatant was discarded by centrifuging, the pellets were dissolved in 100 μl isopropanol containing 0.8 M HCl, and then, the absorbance was measured at 570 nm by using a Bauty Diagnostic Microplate Reader (Molecular Devices, CA, USA).

TUNEL assay for apoptotic DNA fragmentation – The commercially available in situ cell death detection kit (Boehringer Mannheim, Mannheim) was utilized to identify the DNA fragmentation. PC12 cells were seeded at a density 1×10^5 cells/cm^2 on cover slips coated with poly-L-lysine and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. The cells were then washed and refixed in 70% ethanol at 4°C for 30 min. After washed with PBS, the cells were permeabilized with 0.1% triton X-100 in 0.1% sodium acetate for 5 min at 4°C and incubated with 50 μl/well TUNEL reaction mixture for 60 min at 37°C in the dark humidified chamber. After washed with PBS, the cells were exposed to 0.3 ml DNA staining solution (0.1 mM EDTA, PH 7.4, 50 μg/ml RNase A and 50 μg/ml PI) for 30 min at room temperature. The cells were then washed twice with PBS and mounted with 50% glycerol. Stained cells were examined with an Olympus fluorescence upright microscope (Bio-Rad, Herts, UK).

Flow cytometric analysis of apoptotic cells – Cell death was determined by flow cytometry. PC12 cells were harvested by centrifugation and washed in PBS. The cells were fixed in 70% ethanol for 30 min at 4°C. Before staining with PI (50 μg/ml), the fixed cells were again centrifuged and washed in PBS. The cellular DNA content was measured using a FACS vantage fluorescence-activated flow cytometer (Bekton Dickinson, San Jose, CA, USA). Calculation of the percentage of apoptotic cells was based on the cumulative frequency curves of the appropriate DNA histograms.

Statistical analysis – All data were expressed as means±SEM of at least four or five experiments. Statistical analysis was performed using the ANOVA followed by Tukey's test.

Results

Exposure of PC12 cells to (1R,9S)-β-hydrastine hydrochloride at concentrations higher than 500 μM appeared to be cytotoxic when it was examined by MTT assay (Fig. 1A, B). A significant decrease in cell viability was observed when PC12 cells were exposed to L-DOPA at concentrations higher than 50 μM for 48 h while L-DOPA at concentrations of 20 and 50 μM did not affect cell viability after 24 and 48 h treatment (Fig. 1A, B).

In assessing whether (1R,9S)-β-hydrastine hydrochloride could stimulate the cytotoxicity of L-DOPA, we added non-cytotoxic or cytotoxic concentrations of (1R,9S)-β-hydrastine hydrochloride with L-DOPA to the media. When non-cytotoxic concentrations up to 250 μM of (1R,9S)-β-hydrastine hydrochloride were associated with L-DOPA (20, 50, and 100 μM) for 24 h or 48 h, the decrease in cell viability was not observed at both incubation time points. PC12 cells exposure to cytotoxic concentrations of (1R,9S)-β-hydrastine hydrochloride (250, 500, and 750 μM) in association with L-DOPA (20, 50, and 100 μM) at both incubation time points resulted in a significantly decrease in cell viability compared with L-DOPA alone (Fig. 1A, B). Under these conditions, it was noted that high concentrations of (1R,9S)-β-hydrastine hydrochloride enhanced

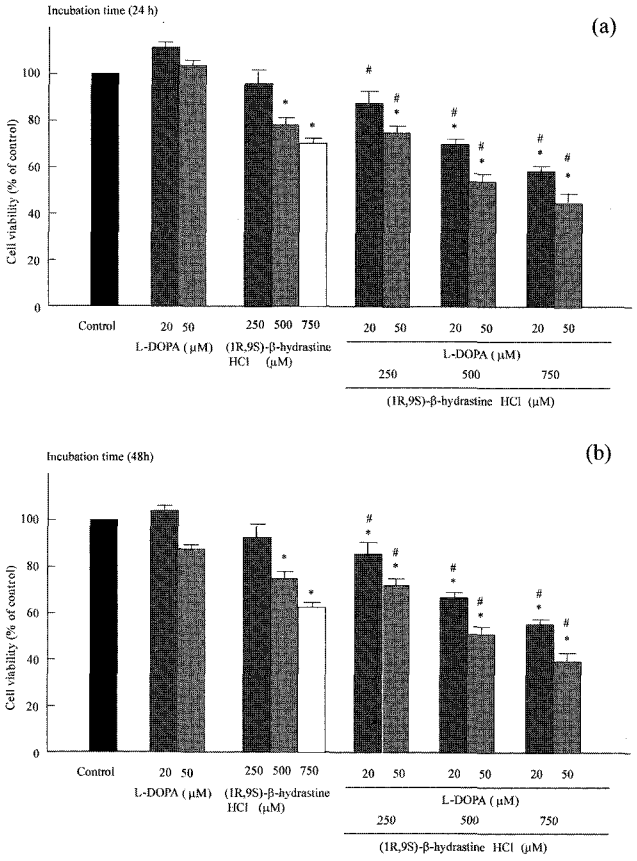

Fig. 1. Effects of (1R,9S)-β-hydrastine hydrochloride on L-DOPA-induced cell viability in PC12 cells. PC12 cells were exposed to L-DOPA (20, 50, and 100 μM) in the absence or presence of (1R,9S)-β-hydrastine hydrochloride (250, 500, and 750 μM) for 24 h (A) or 48 h (B), and the cell viability was assessed by the MTT method, in which viable cells convert the soluble dye, MTT to in soluble blue formazan crystals. Results represent the means±SEM of five experiments performed in triplicate. *, $p<0.05$ compared with the control; #, $p<0.05$ compared with the corresponding L-DOPA concentrations (ANOVA followed by Tukey's test).

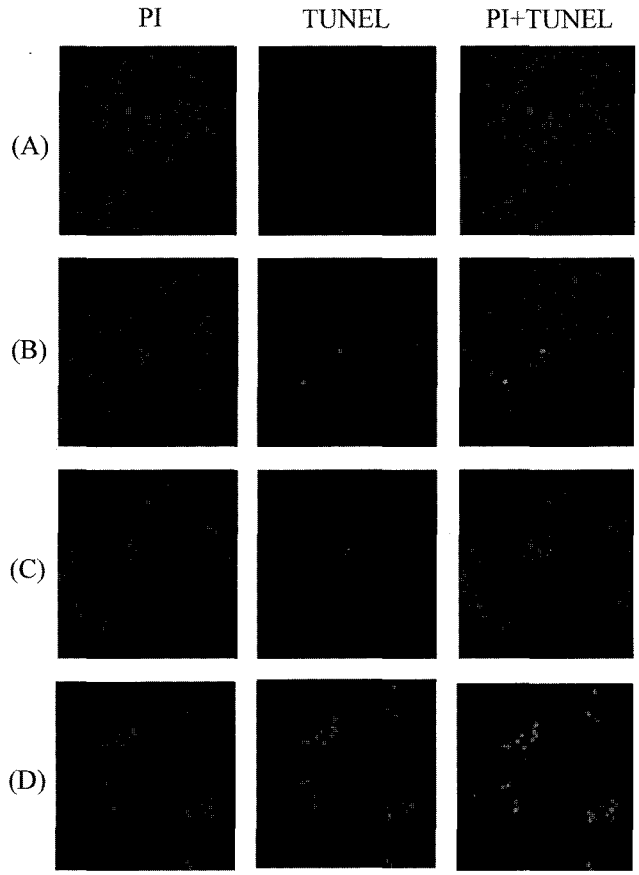

Fig. 2. Synergistic effects of (1R,9S)-β-hydrastine hydrochloride on L-DOPA-induced apoptosis in PC12 cells as determined by *in situ* TUNEL. Fluorescence micrographs of untreated PC12 cells (A) and apoptotic PC12 cells (green or yellow green cells) after 48 h exposed to (1R,9S)-β-hydrastine hydrochloride 750 μM (B), L-DOPA 50 μM (C), L-DOPA 50 μM + (1R,9S)-β-hydrastine hydrochloride 750 μM (D). PI was used to counterstain the cells. Apoptotic nuclei are those with green or yellow-green fluorescence.

the loss of cell viability compared with the untreated control, and that exposure to high concentration of (1R,9S)-β-hydrastine hydrochloride plus L-DOPA for 24 h or 48 h resulted in a marked reduction in the cell viability; exposure for 48 h was more cytotoxic than that of 24 h.

To examine whether the cellular damage induced by (1R,9S)-β-hydrastine hydrochloride plus L-DOPA might be a characteristic of changes leading to apoptosis, we used the TUNEL technique and flow cytometry. When cytotoxic concentrations of (1R,9S)-β-hydrastine hydrochloride (750 μM) were associated with L-DOPA (50 μM) for 48 h, the percentage of apoptotic cells was markedly increased compared with the cells treated with L-DOPA alone (Fig. 2). TUNEL technique revealed that the treatment with 750 μM of (1R,9S)-β-hydrastine hydrochloride plus 50 μM L-DOPA for 48 h stimulated cell death via a mechanism, which possessed characteristic morphological features of apoptotic cell death, including highly condensed chromatin and extensive membrane blebbing (Fig. 2). The percentage of apoptotic cells after incubation with (1R,9S)-β-hydrastine hydrochloride, as revealed by flow cytometry, was increased in a concentration-dependent manner. When a cytotoxic concentration of (1R,9S)-β-hydrastine hydrochloride (750 μM) were associated with L-DOPA (50 μM) for 24 h (data not shown) or 48 h (Fig. 3), the percentage of apoptotic cells was also increased compared with that of the cells treated with L-DOPA alone.

Discussion

IQs were first characterized as inhibitors of TH activity (Nagatsu and Hirata, 1997) and have now been reported to possess many of the cytotoxic characteristic of MPTP

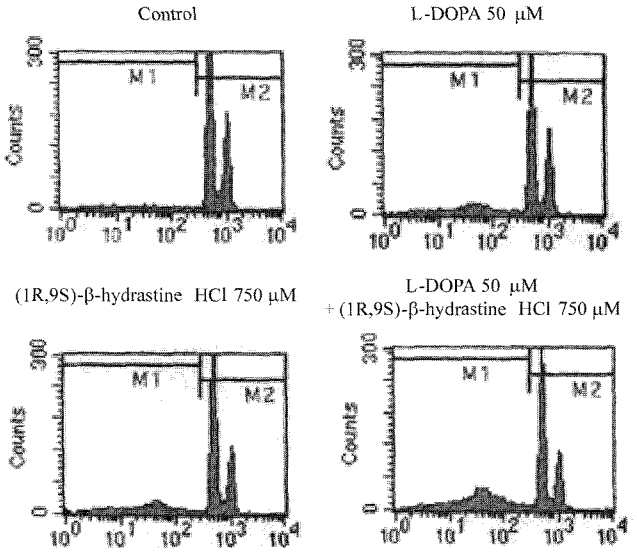

Fig. 3. Flow cytometry histograms of control PC12 cells and PC12 cells after 48 h exposure to (1R,9S)-β-hydrastine hydrochloride (750 μM) alone or associated with L-DOPA (50 μM). After incubation, the cells were harvested and stained with PI. DNA relative content was analyzed by flow cytometry. X-axis, DNA content; Y-axis, number of cells.

(Desole *et al.*, 1996; Przedborski and Vila, 2001). L-DOPA itself has been shown to be toxic to cells in culture because of its ability to be spontaneously oxidized, resulting in formation of quinones and free radicals (Basma *et al.*, 1995). Previously, (1R,9S)-β-hydrastine hydrochloride in lower concentrations of 10-50 μM inhibits dopamine biosynthesis in PC12 cells (Kim *et al.*, 2001). Tetrahydropapaveroline, one of TIQ alkaloids, is proposed to have an oxidative stress-induced cytotoxicity and enhances L-DOPA-induced cytotoxicity in PC12 cells (Lee *et al.*, 2003). Therefore, the present study was designed to investigate whether (1R,9S)-β-hydrastine hydrochloride could worsen L-DOPA cytotoxicity.

L-DOPA at concentrations higher than 50 μM led to cell damage, and that the degree of cell damage was proportioned by the incubation time (Fig. 1A, B). The cytotoxic concentration of L-DOPA (50 μM) used in the present study was about 5-times higher than the therapeutic concentrations in Parkinsonian patients (Rossor *et al.*, 1980). The cytotoxic action of L-DOPA is its autoxidation into reactive toxic free radicals such as hydroxyl radicals, oxygen free radicals and various quinone derivatives (Graham *et al.*, 1978; Rosenberg, 1988). Media containing L-DOPA turned to an orange-brownish color with time in culture, and this color is an indicative of the presence of quinines (Basma *et al.*, 1995). The same color change was also observed in the media containing L-DOPA in the present study. However, the possibility that quinines formed from the autoxidation of L-DOPA are actual mediators of toxicity was not directly tested.

In this study, both (1R,9S)-β-hydrastine hydrochloride (500-750 μM) and L-DOPA (50 μM) at cytotoxic concentrations exhibited morphological characteristics of apoptosis such as cell shrinkage and membrane blebbing (Fig. 2). In addition, concurrent incubation of the cells with (1R,9S)-β-hydrastine hydrochloride and L-DOPA produced a greater effect on cytotoxicity over (1R,9S)-β-hydrastine hydrochloride or L-DOPA alone in a concentration- and time-dependent manner (Fig. 1, 2, 3).

On the basis of above results, (1R,9S)-β-hydrastine hydrochloride at cytotoxic concentrations (500-750 μM) enhances L-DOPA-induced cytotoxicity in PC12 cells. Furthermore, the possibilities that the patients on long-term L-DOPA therapy are potentially at risk of the exogenous neurotoxicants such as TIQs including (1R,9S)-β-hydrastine hydrochloride are proposed.

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