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Effects of (1*R*,9*S*)-β-hydrastine on L-DOPA-induced cytotoxicity in PC12 cells

Shou Yu Yin[a,b], Jae Joon Lee[a], Yu Mi Kim[a], Chun Mei Jin[a], Yoo Jung Yang[a], Min Hee Kang[a], Masaaki Kai[c], Myung Koo Lee[a,*]

[a] *College of Pharmacy, and Research Center for Bioresource and Health, Chungbuk National University, San 48, Kaeshin-Dong, Heungduk-Gu, Cheongju 361-763, South Korea*
[b] *College of Pharmacy, Yanbian University, 121 Juzi Street, Yanji, Jilin, 133000, PR China*
[c] *School of Pharmaceutical Sciences, Nagasaki University, Bunkyo-Machi 1-14, Nagasaki 852-8521, Japan*

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Abstract

(1*R*,9*S*)-β-Hydrastine in lower concentrations of 10–50 μM inhibits dopamine biosynthesis in PC12 cells (Planta Med. 57 (2001) 609). In this study, the effects of (1*R*,9*S*)-β-hydrastine on L-DOPA (L-3,4-dihydroxyphenylalanine)-induced cytotoxicity in PC12 cells were investigated. (1*R*,9*S*)-Hydrastine at concentrations up to 250 μM did not reduce cell viability. However, at concentrations higher than 500 μM it caused cytotoxicity in PC12 cells, as determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, TUNEL (terminal deoxynucleotidyltransferase dUTP nick-end labeling) method and flow cytometry. Exposure of PC12 cells to cytotoxic concentrations of (1*R*,9*S*)-β-hydrastine (500 and 750 μM) in combination with L-DOPA (20, 50 and 100 μM) after 24 or 48 h resulted in a significant decrease in cell viability compared with the effects of (1*R*,9*S*)-β-hydrastine or L-DOPA alone, and apoptotic cell death was observed. However, the decrease in cell viability induced by (1*R*,9*S*)-β-hydrastine was not prevented by the antioxidant *N*-acetyl-L-cysteine, indicating that it is not mediated by membrane-based oxygen free radical damage. These data suggest that (1*R*,9*S*)-β-hydrastine has a mild cytotoxic effect and at higher concentration ranges aggravates L-DOPA-induced cytotoxicity in PC12 cells.

Keywords: (1*R*,9*S*)-β-Hydrastine; L-DOPA (L-3,4-dihydroxyphenylalanine); Cytotoxicity; PC12 cell; Apoptosis

1. Introduction

Hydrastine derivatives are composed of a phthalide and an isoquinoline alkaloid and exist in two configurations, such as (1*R*,9*S*)-β-hydrastine [(−)-β-hydrastine] and (1*S*,9*R*)-β-hydrastine [(+)-β-hydrastine] (Fig. 1). Concerning the pure base, the (1*R*,9*S*)-form is a well-known component of the roots of *Hydrastis canadensis* L. (Ranunculaceae) and *Berberis laurina* Billb. (Berberidaceae), and the (1*S*,9*R*)-form occurs in the roots of *Corydalis stricta* Steph. (Papaveraceae) (Stanek and Manske, 1968; Tang and Eisenbrand, 1992). (1*S*,9*R*)-β-Hydrastine has an antagonistic effect on gamma-aminobutyric acid receptors (Huang and Johnston, 1990). (1*R*,9*S*)-β-Hydrastine has been found to inhibit competitively bovine adrenal tyrosine hydroxlase (EC 1.14.16.2; TH) with L-tyrosine as a substrate (Lee et al., 1997). Recently, (1*R*,9*S*)-β-hydrastine, but not (1*S*,9*R*)-β-hydrastine, at lower concentrations of 10–50 μM was found to inhibit dopamine biosynthesis, in part through the inhibition of TH activity in PC12 cells (Kim et al., 2001).

Many studies have confirmed that tetrahydroisoquinolines are linked to Parkinson's disease and brain damage due to chronic alcoholism (Kotake et al., 1995; Maruyama et al., 1996). The underlying mechanisms of tetrahydroisoquinoline-induced apoptosis are mediated by oxidative stress and mitochondrial energy depletion (Seaton et al., 1997; Morikawa et al., 1998). Hydrastine derivatives have a similar tetrahydroisoquinoline configuration, and so it is conceivable that hydrastine derivatives might cause neurodegeneration. However, the cytotoxicity of hydrastine derivatives, especially (1*R*,9*S*)-β-hydrastine, has not been examined even though in spite (1*R*,9*S*)-β-hydrastine has inhibitory activity on dopamine biosynthesis.

* Corresponding author. Tel.: +82-43-261-2822; fax: +82-43-276-2754.
E-mail address: myklee@cbucc.chungbuk.ac.kr (M.K. Lee).

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L-DOPA (L-3,4-dihydroxyphenylalanine) is the most frequently prescribed drug for controlling the symptoms of Parkinson's disease (Marsden, 1994), due to its ability to raise the dopamine level in the striatum (Hornykiewicz, 1994). However, some reports have suggested that L-DOPA may accelerate deterioration of the condition of Parkinsonian patients and that L-DOPA toxicity occurs in damaged dopamine neurons in vivo (Boyce et al., 1990). It is also reported that L-DOPA produces neurotoxic reactive oxygen species, leading to apoptosis due to autoxidation and enzymatic oxidation (Sandstrom et al., 1994).

The pheochromocytoma, PC12, cell lines were originally characterized from a catecholamine-secreting adrenal chromaffin tumor in rats (Greene and Tischler, 1976) and have been widely used as in vitro models to investigate dopaminergic toxicity, such as L-DOPA neurotoxicity, L-DOPA autoxidation, oxidative stress and mitochondrial impairment (Itano et al., 1994; Basma et al., 1995; McNaught et al., 1996; Migheli et al., 1999).

In this study, therefore, the cytotoxic effects of (1*R*,9*S*)-β-hydrastine, alone or in combination with L-DOPA, in PC12 cells were investigated. In addition, we also examined the role of the antioxidant *N*-acetyl-L-cysteine in the cytotoxicity induced by (1*R*,9*S*)-β-hydrastine.

2. Materials and methods

2.1. Chemicals

(1*R*,9*S*)-β-Hydrastine, L-DOPA, ribonuclease A (Rnase A), propidium iodide, ethylenediaminetetraacetic acid (EDTA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). The in situ cell death detection kit (TUNEL: terminal deoxynucleotidyltransferase dUTP nick-end labeling) was supplied by 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.

2.2. 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. PC12 cells (ca. 1×10^5 cells/cm^2) were incubated in the absence or presence of L-DOPA (20–100 μM) together with (1*R*,9*S*)-β-hydrastine in concentrations ranging from 50 to 750 μM for 24 or 48 h.

2.3. Assessment of cell viability

Cell viability was determined with the conventional MTT assay with a slight modification (Mosman, 1983). The assay is based on the conversion of tetrazolium salt into an insoluble formazan product by various dehydrogenases in mitochondria. PC12 cells were diluted to 1×10^5 cells/ml and 100 μl was added to the wells of a 96-well microplate. PC12 cells were treated with various concentrations of (1*R*,9*S*)-β-hydrastine (50–750 μM) and L-DOPA (20–100 μM), alone or in combination, for 24 or 48 h. The MTT solution (final concentration 1 mg/ml) was added to the cells and the cultures were allowed to incubate at 37 °C for 3–4 h. After centrifugation, the supernatant was discarded, 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). Cell viability is expressed as percentage of the control value.

2.4. TUNEL assay for apoptotic DNA fragmentation

The commercially available in situ cell death detection kit (Boehringer Mannheim, Mannheim) was used to identify DNA fragmentation. PC12 cells were seeded at a density of 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 being 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 a dark humidified chamber. After being 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 propidium iodide) 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).

2.5. Flow cytometric analysis of apoptotic cells

PC12 cells were harvested, washed with ice-cold PBS, fixed in ice-cold 70% ethanol and stored at −20 °C until analysis. The cells were then washed in PBS before being suspended in 1 ml staining solution (50 μg/ml propidium iodide, 0.05 mg/ml Rnase A and 0.1mM EDTA, pH 7.4 in PBS) and incubated at room temperature for 30 min. Analysis of 10,000 events was performed on a FACScan flow cytometer and the percentage of apoptotic cells was calculated, based on the cumulative frequency curves of the appropriate DNA histograms. Flow cytometric diagrams were recorded using a FACS vantage fluorescence-activated flow cytometer (Bekton Dickinson, San Jose, CA, USA).

2.6. Statistical analysis

All data are expressed as means ± S.E.M. of at least four or five experiments. Statistical analysis were per-

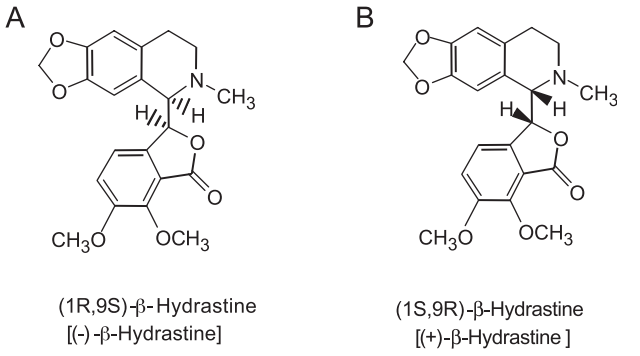

Fig. 1. Chemical structures of (1*R*,9*S*)-β-hydrastine (A) and (1*S*,9*R*)-β-hydrastine (B).

formed using the ANOVA (analysis of variance) followed by Tukey′ s test.

3. Results

3.1. Inhibition of cell viability by (1R,9S)-β-hydrastine and L-DOPA

(1*R*,9*S*)-β-Hydrastine at concentrations up to 250 μM did not significantly reduce cell viability in PC12 cells. However, when PC12 cells were treated with 500–750 μM (1*R*,9*S*)-β-hydrastine for 24 or 48 h, there was a concentration- and time-dependent reduction in cell viability, as determined with the MTT assay (Fig. 2). Cytotoxicity was greater when (1*R*,9*S*)-β-hydrastine was added to PC12 cells for 48 h instead of 24 h (Fig. 2).

L-DOPA at concentrations of 20 and 50 μM did not significantly decrease cell viability after 24 or 48 h compared with the untreated control (Fig. 3A,B). However, a significant decrease in cell viability was observed when PC12 cells were treated with L-DOPA at concentrations higher than 50 μM for 48 h (Fig. 3B).

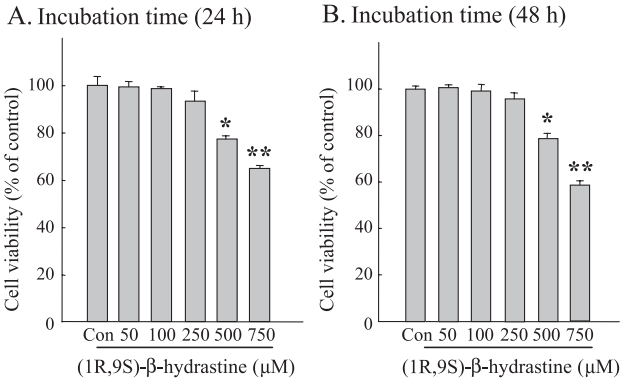

Fig. 2. Effects of (1*R*,9*S*)-β-hydrastine on PC12 cell viability. PC12 cells were exposed for 24 h (A) or 48 h (B) to various concentrations of (1*R*,9*S*)-β-hydrastine (50–750 μM). Cell viability was assessed using the MTT method, in which viable cells convert the soluble dye, MTT, to insoluble blue formazan crystals. The results represent the means ± S.E.M. of five experiments performed in triplicate. *$P<0.05$, **$P<0.01$ compared with the control (ANOVA followed by Tukey's test).

3.2. Enhancing effects of (1R,9S)-β-hydrastine on L-DOPA-induced cytotoxicity

To assess whether (1*R*,9*S*)-β-hydrastine could aggravate the cytotoxicity of L-DOPA, non-cytotoxic or cytotoxic concentrations of (1*R*,9*S*)-β-hydrastine were added with L-DOPA to the medum. When (1*R*,9*S*)-β-hydrastine at a non-cytotoxic concentration of 250 μM was added with L-DOPA (50 and 100 μM) for 24 or 48 h, a decrease in cell viability was observed at both incubation time points, while hydrastine concentrations lower than 250 μM did not affect cell viability when added together with L-DOPA (20, 50 and 100

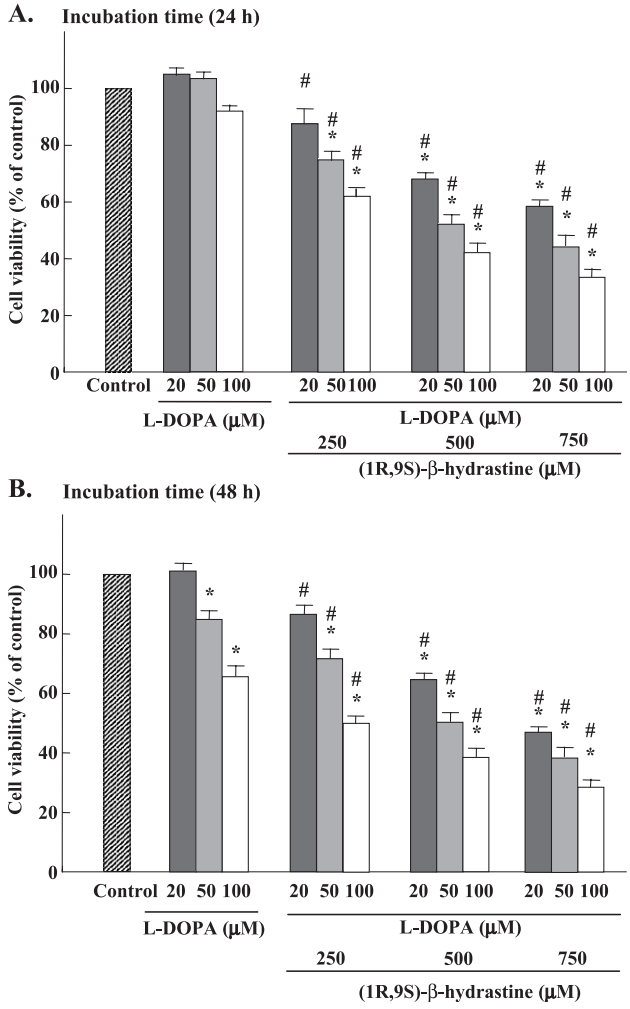

Fig. 3. Effects of (1*R*,9*S*)-β-hydrastine on L-DOPA-induced cell viability in PC12 cells. PC12 cells were treated with L-DOPA (20, 50 and 100 μM) in the absence or presence of (1*R*,9*S*)-β-hydrastine (250, 500 and 750 μM) for 24 h (A) or 48 h (B), and cell viability was assessed with the MTT method, in which viable cells convert the soluble dye, MTT, to insoluble blue formazan crystals. Results represent the means ± S.E.M. 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).

μM) (data not shown). In addition, exposure of PC12 cells to cytotoxic concentrations of (1*R*,9*S*)-β-hydrastine (500 and 750 μM) in combination with L-DOPA (20, 50 and 100 μM) at both incubation time points resulted in a marked decrease in cell viability compared with the effects of L-DOPA alone (Fig. 3). Under these conditions, it was noted that higher concentrations of (1*R*,9*S*)-β-hydrastine enhanced the loss of cell viability compared with the untreated control, and that exposure to higher concentrations of (1*R*,9*S*)-β-hydrastine plus L-DOPA for 24 or 48 h resulted in a marked reduction in cell viability.

In addition, neither (1*R*,9*S*)-β-hydrastine (50–250 μM) nor L-DOPA (20 μM) induced nuclear changes characteristic of apoptosis at 48 h in PC12 cells, according to the TUNEL assay (data not shown). However, (1*R*,9*S*)-β-hydrastine at 750 μM, L-DOPA at 50 μM and (1S,9R)-β-hydrastine (750 μM) in combination with L-DOPA (50 μM) after 48 h induced apoptotic nuclear changes (Fig. 3). The percentage of apoptotic cells after incubation with (1*R*,9*S*)-β-hydrastine, as revealed by flow cytometry, was increased in a concentration-dependent manner. When a cytotoxic concentration of (1*R*,9*S*)-β-hydrastine (750 μM) was combined with L-DOPA (50 μM) for 24 h (data not shown) or 48 h (Fig. 5), the percentage of apoptotic cells was also increased compared with that of the cells treated with L-DOPA alone.

3.3. Effects of N-acetyl-L-cysteine on (1R,9S)-β-hydrastine or L-DOPA cytotoxicity

To evaluate the role of oxidative stress, the effects of antioxidant *N*-acetyl-L-cysteine on (1*R*,9*S*)-β-hydrastine- or L-DOPA-induced cell death in PC12 cells were examined. *N*-acetyl-L-cysteine (0.2 mM), when added to the cell cultures, partially inhibited the L-DOPA-induced decrease in cell viability at concentrations of 50 and 100 μM, as determined with the MTT assay (Fig. 6). However, *N*-acetyl-L-cysteine failed to reduce (1*R*,9*S*)-β-hydrastine (750–1500 μM)-induced cytotoxicity (Fig. 6).

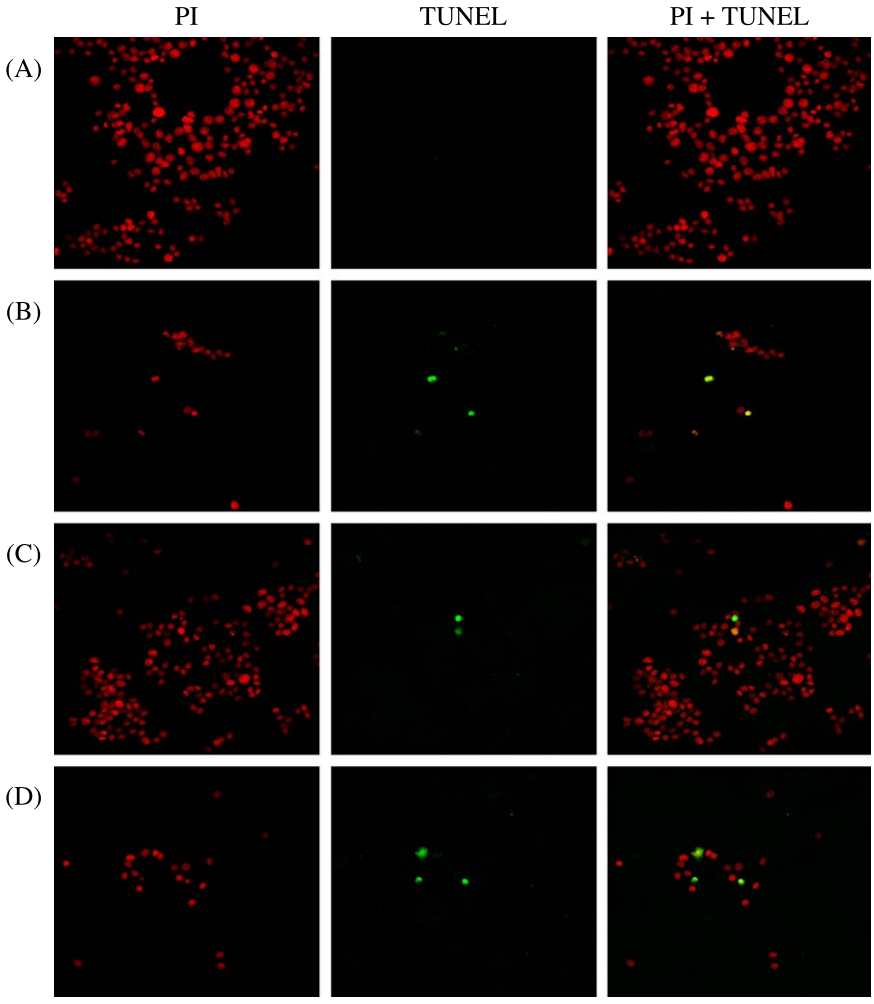

Fig. 4. Synergistic effects of (1*R*,9*S*)-β-hydrastine on L-DOPA-induced apoptosis in PC12 cells as determined by in situ TUNEL. Fluorescence micrographs of untreated PC12 cells (A) and apoptotic PC12 cells (green or yellow green cells) after 48-h incubation with (1*R*,9*S*)-β-hydrastine 750 μM (B), L-DOPA 50 μM (C), L-DOPA 50 μM+(1*R*,9*S*)-β-hydrastine 750 μM (D). Propidium iodide (PI) was used to counterstain the cells. Apoptotic nuclei are those with green or yellow-green fluorescence.

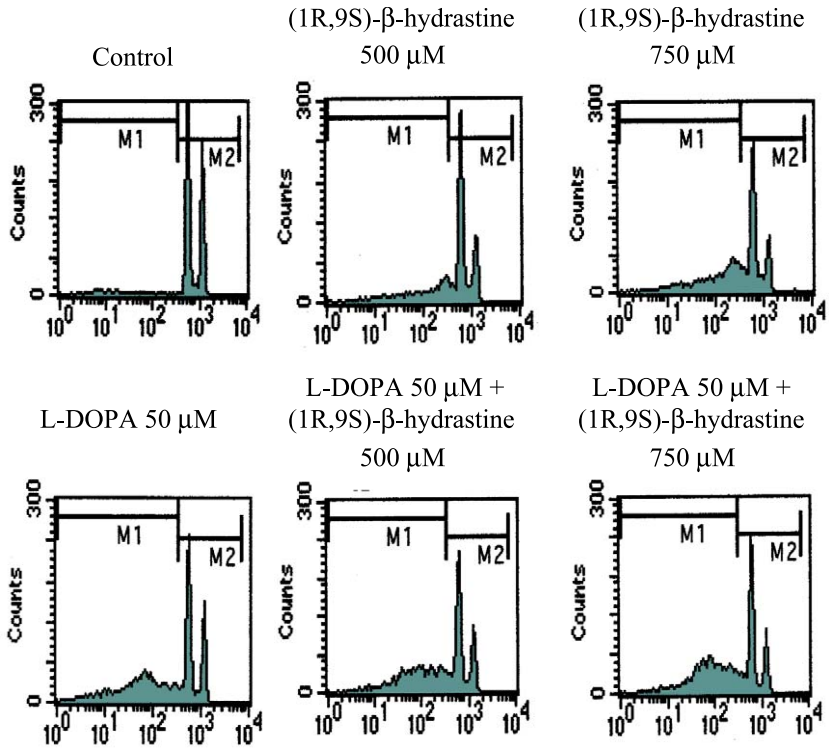

Fig. 5. Flow cytometry histograms of control PC12 cells and PC12 cells after a 48-hour incubation with (1*R*,9*S*)-β-hydrastine (500 and 750 μM) alone or in combination with L-DOPA (50 μM). After incubation, the cells were harvested and stained with propidium iodide. DNA relative content was analyzed by flow cytometry. *X*-axis, DNA content; *Y*-axis, number of cells.

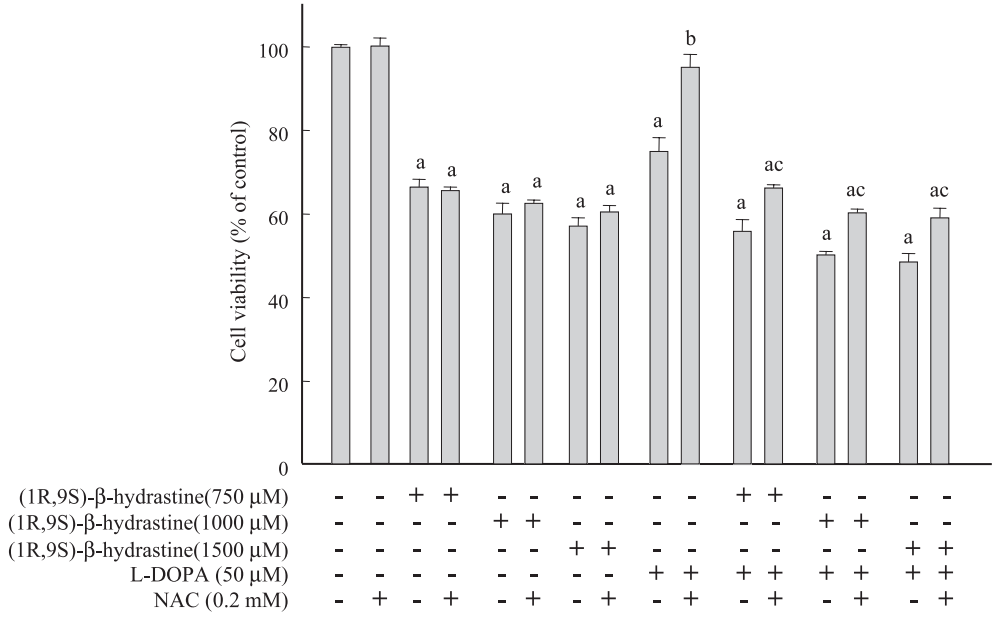

Fig. 6. Effects of *N*-acetyl-L-cysteine on (1*R*,9*S*)-β-hydrastine, L-DOPA and (1*R*,9*S*)-β-hydrastine plus L-DOPA-induced decrease in PC12 cell viability after 24 h. PC12 cells were cultured in RPMI 1640 medium with (1*R*,9*S*)-β-hydrastine (750–1500 μM), L-DOPA (50 μM) and (1*R*,9*S*)-β-hydrastine plus L-DOPA only or in combination with 0.2 mM *N*-acetyl-L-cysteine. The results represent the means ± S.E.M. of five experiments performed in triplicate. (a) $P < 0.05$ compared with control; (b) $P < 0.05$ compared with 50 μM L-DOPA; (c) $P < 0.05$ compared with corresponding (1*R*,9*S*)-β-hydrastine + L-DOPA concentrations (ANOVA followed by Tukey's test).

4. Discussion

Tetrahydroisoquinolines are inhibitors of TH activity, the rate-limiting enzyme of the catecholamine biosynthetic pathway (Nagatsu and Hirata, 1987), and have been reported to possess many of the cytotoxic characteristics of 1-methyl-4-phenyl-1,2,3,6-tetrahydrapyridine (MPTP), which causes a Parkinson-like syndrome in human and non-human primates (Tasaki et al., 1991; Desole et al., 1996; McNaught et al., 1998; Ohta, 2002; Przedborski and Vila, 2001). L-DOPA itself has been shown to be toxic to PC12 cells in culture because of its ability to be spontaneously oxidized, resulting in the formation of quinones and free radicals (Basma et al., 1995). Recently, (1*R*,9*S*)-β-hydrastine at low concentrations of 10–50 μM decreased the dopamine content in PC12 cells by inhibiting TH activity and TH gene expression (IC_{50} value, 20.7 μM) (Kim et al., 2001). In addition, the increase in dopamine levels induced by L-DOPA (20–100 μM) in PC12 cells was in part inhibited when L-DOPA was combined with 10–50 μM (1*R*,9*S*)-β-hydrastine (data not shown). In this study, the apoptotic effects of (1*R*,9*S*)-β-hydrastine on L-DOPA-induced cytotoxicity in PC12 cells were investigated.

(1*R*,9*S*)-β-Hydrastine at 500–750 μM caused a dose-dependent cytotoxic effect, as measured with the MTT assay, in PC12 cells (Fig. 2). Concurrent incubation of the cells with (1*R*,9*S*)-β-hydrastine (500–750 μM) and L-DOPA (50 μM) produced a greater cytotoxic effect than (1*R*,9*S*)-β-hydrastine or L-DOPA alone in a concentration-dependent manner (Fig. 3). In addition, after a 48-h incubation with both (1*R*,9*S*)-β-hydrastine (500–750 μM) and L-DOPA (20–100 μM) at cytotoxic concentrations, PC12 cells exhibited the characteristics of apoptosis, as determined by TUNEL assay and flow cytometry analysis (Figs. 4 and 5). Therefore, these results indicate that (1*R*,9*S*)-β-hydrastine at cytotoxic concentrations of 500–750 μM enhances L-DOPA-induced cytotoxicity in PC12 cells.

Oxidative stress is considered a mediator of L-DOPA-induced apoptosis (Migheli et al., 1999). In addition, the fact that antioxidants such as *N*-acetyl-L-cysteine, vitamin E, glutathione and ascorbic acid inhibit L-DOPA-induced apoptosis in PC12 cells (Walkinshaw and Waters, 1995) supports the hypothesis that oxidative stress is involved as a mediator of L-DOPA-induced apoptosis. In agreement with previous studies (Lee et al., 2003), L-DOPA-induced cytotoxicity was inhibited by the antioxidant *N*-acetyl-L-cysteine (Fig. 6), suggesting that activation of apoptosis is mediated by oxygen free radicals. However, in this study, *N*-acetyl-L-cysteine failed to inhibit the (1*R*,9*S*)-β-hydrastine-induced decrease in cell viability (Fig. 6). These results indicate that mitochondrial dysfunction and intracellular energy metabolism may play greater roles than oxygen free radicals in the mechanism of cell death induced by (1*R*,9*S*)-β-hydrastine in PC12 cells.

Parkinsonian patients have deficits in NADH CoQ reductase (Schapira et al., 1990), cytochrome *c* oxidase (Itoh et al., 1997) and α-ketoglutarate dehydrogenase (Mizuno et al., 1995). Some tetrahydroisoquinoline compounds inhibit mitochondrial respiration and NADH CoQ reductase (McNaught et al., 1996, 1998). Therefore, it is possible that the mechanism responsible for the activation of apoptosis by (1*R*,9*S*)-β-hydrastine, similar to that for tetrahydroisoquinoline-induced apoptosis, is in part associated with an impairment of energy metabolism in PC12 cells. This needs to be studied further.

On the basis of the above results, (1*R*,9*S*)-β-hydrastine at concentrations higher than 500 μM showed a mild cytotoxic effect and enhanced L-DOPA-induced apoptosis in PC12 cells. The present study also indicates that the mechanism of intracellular death induced by (1*R*,9*S*)-β-hydrastine is most likely not related to the destructive effects of oxygen free radicals. However, (1*R*,9*S*)-β-hydrastine at low concentrations of 10–50 μM inhibited dopamine biosynthesis in PC12 cells. The cytotoxic concentration range of (1*R*,9*S*)-hydrastine was about 25-fold higher than the IC_{50} value (20.7 μM) obtained for dopamine biosynthesis in PC12 cells. Therefore, (1*R*,9*S*)-hydrastine regulates dopamine biosynthesis, showing weak cytotoxicity in PC12 cells. The pharmacological functions of (1*R*,9*S*)-hydrastine in relation to catecholamine biosynthesis will be studied further.

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