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Biochemical Pharmacology 66 (2003) 1787–1795
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Aggravation of L-DOPA-induced neurotoxicity by tetrahydropapaveroline in PC12 cells

Jae Joon Lee, Yu Mi Kim, Shou Yu Yin, Hee Dong Park, Min Hee Kang, Jin Tae Hong, Myung Koo Lee*

College of Pharmacy, and Research Center for Bioresource and Health, Chungbuk National University, San 48, Kaeshin-Dong, Heungduk-Ku, Cheongju 361-763, South Korea

Received 21 February 2003; accepted 17 June 2003

Abstract

Tetrahydropapaveroline (THP) is formed in Parkinsonian patients receiving L-DOPA therapy and is detected in the plasma and urine of these patients. In this study, we have investigated the effects of THP on L-DOPA-induced neurotoxicity in cultured rat adrenal pheochromocytoma, PC12 cells. Exposure of PC12 cells up to 10 μM THP or 20 μM L-DOPA after 24 or 48 hr, neither affected the cell viability determined by MTT assay, nor induced apoptosis by flow cytometry and TUNEL staining. However, at concentrations higher than 15 μM, THP showed cytotoxicity through an apoptotic process. In addition, THP at 5–15 μM for both incubation time points significantly enhanced L-DOPA-induced neurotoxicity (L-DOPA concentration, 50 μM). Exposure of PC12 cells to THP, L-DOPA and THP plus L-DOPA for 48 hr resulted in a marked increase in the cell loss and percentage of apoptotic cells compared with exposure for 24 hr. The enhancing effects of THP on L-DOPA-induced neurotoxicity were concentration- and treated-time-dependent. THP, L-DOPA and THP plus L-DOPA produced a significant increase in intracellular reactive oxygen species generation and decrease in ATP levels, supporting the involvement of oxidative stress in THP- and L-DOPA-induced apoptosis. The antioxidant *N*-acetyl-L-cysteine strongly inhibited changes in apoptosis, decreases in cell viability and ROS generation induced by THP associated with L-DOPA. These results suggest that THP aggravates L-DOPA-induced oxidative neurotoxic and apoptotic effects in PC12 cells. Therefore, Parkinsonian patients treated with L-DOPA for long-term need to be monitored for the relationship between plasma concentration of THP and the symptoms of neurotoxicity.

Keywords: Tetrahydropapaveroline; L-DOPA; Neurotoxicity; PC12 cells; Apoptosis; Oxidative stress

1. Introduction

Parkinson's disease is a common, slowly progressing movement disorder accompanied by tremor, muscle rigidity and akinesia. It is mainly due to a deficiency of dopamine as the result of degeneration and cell death of the nigrostriatal dopaminergic neurons [1]. The etiology of this disease remains unknown, but one hypothesis is that endogenous or exogenous neurotoxins may be responsible for the destruction of nigrostriatal neurons.

Tetrahydropapaveroline [6,7-dihydroxy-(3′,4′-dihydroxybenzyl)-1,2,3,4- tetrahydroisoquinoline, THP] is the condensation product of dopamine and its aldehyde-metabolite (3,4-dihydroxyphenylacetaldehyde) formed by catalysis of monoamine oxidase. THP has been identified in the urine, brain and plasma of Parkinsonian patients receiving oral L-DOPA therapy [2], and in the brain of animals treated with L-DOPA [3] or ethanol [4]. THP and its alkaloids have been proposed to be candidates of dopaminergic neurotoxins related to the pathogenesis of Parkinsonism [5,6]. Oxidative stress [7] and mitochondrial energy depletion [8,9] have been thought as the underlying mechanisms of THP-induced apoptosis. However, despite endogenous formation and potential human exposure to THP, little is known about the biochemical mechanism underlying the adverse effects of the neurotoxic compound.

The pheochromocytoma, PC12, cell lines are originally characterized from a catecholamine-secreting adrenal

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

Abbreviations: $DCFH_2$-DA, 2′,7′-dichlorofluorescin diacetate; L-DOPA, L-3,4-dihydroxyphenylalanine; NAC, *N*-acetyl-L-cysteine; MTT, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PC12 cells, pheochromocytoma cells; PI, propidium iodide; PBS, phosphate-buffered saline; ROS, reactive oxygen species; THP, tetrahydropapaveroline; TUNEL, terminal deoxynucleotidyltransferase.

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doi:10.1016/S0006-2952(03)00421-0

chromaffin tumor in rats [10,11], and have been widely used to investigate neuronal differentiation, signal transduction and neuronal cell death [12].

L-DOPA is the most frequently prescribed drug for controlling the symptoms of Parkinson's disease [13]. The treatment of choice is the administration of L-DOPA, which results in the decarboxylase-catalyzed conversion of L-DOPA to dopamine in the brain catecholaminergic neurons and, hence, in a replenishment of dopamine in the surviving neurons and an alleviation of the symptoms of the disease [13–15]. On the other hand, there are limitations to chronic treatment of L-DOPA, including debilitating side effects and a progressive decrease in efficacy with time [13,16–18]. Furthermore, some reports have suggested that L-DOPA may accelerate the deterioration of Parkinsonian patients [19], and that L-DOPA toxicity occurs in damaged dopaminergic neurons *in vivo* [19–21]. Thus, numerous toxicological studies have utilized PC12 cells as *in vitro* models to examine dopaminergic toxicity such as L-DOPA cytotoxicity, L-DOPA autoxidation, oxidative stress, and mitochondrial impairment [12,22,23].

Therefore, in this study, to see whether THP could in turn worsen L-DOPA neurotoxicity, the enhancing effects of exposure of PC12 cells to increasing concentrations of L-DOPA alone or in combination with non-cytotoxic or cytotoxic concentrations of THP were investigated. In addition, to evaluate the role of oxidative stress in THP-induced apoptosis, we also examined the effects of *N*-acetyl-L-cysteine (NAC) on the cell viability loss and apoptosis in the THP-treated PC12 cells.

2. Materials and methods

2.1. Chemicals

(±)-THP hydrobromide, L-DOPA, RNase A, propidium iodide (PI), poly-L-lysine, NAC and 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from the Sigma. 2′,7′-Dichlorofluorescin diacetate ($DCFH_2$-DA) was obtained from Molecular Probes Inc. Luciferase assay system was purchased from Promega. The *in situ* cell death detection kit (TUNEL) was supplied from the Boehringer Mannheim. All sera, antibiotics and RPMI 1640 for cell culture were obtained from the Gibco. All other chemicals were of reagent grade.

2.2. Cell culture

PC12 cells were maintained routinely [11]. PC12 cells (ca. 1×10^5 cells/cm^2) in culture were incubated in the absence or presence of L-DOPA (20–100 μM) associated with increasing concentrations of THP (5–20 μM) for 24 or 48 hr. NAC concentration was chosen according to Sandstrom *et al*. [24].

2.3. Assessment of cell viability

Cell viability was determined by the conventional MTT assay with slight modification [25], which 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 plated at 100 μL per well in a 96-well microplate. The cells were treated with various concentrations of THP (5–20 μM) and L-DOPA (20–100 μM), alone or in combination, for 24 or 48 hr. After incubation, cells were treated with the MTT solution (final concentration, 1 mg/mL) for 4 hr at 37° in a incubator. The reaction was stopped by adding 100 μL of 0.8 M HCl in isopropanol, and the absorbance was measured at 570 nm by using a Bauty Diagnostic Microplate Reader (Molecular Devices).

2.4. 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°. 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). Calculation of the percentage of apoptotic cells was based on the cumulative frequency curves of the appropriate DNA histograms.

2.5. TUNEL assay for apoptotic DNA fragmentation

The commercially available *in situ* cell death detection kit (Boehringer Mannheim) was utilized to detect DNA fragmentation. For nicked DNA end-labeling experiments, PC12 cells were plated at a density 1×10^5 cells/cm^2 on cover slips coated with poly-L-lysine, and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. The cells were then permeabilized with 0.1% triton X-100 in 0.1% sodium acetate for 5 min at 4°, and incubated with 50 μL/well TUNEL reaction mixture for 60 min at 37° in a CO_2 incubator. The cells were rinsed with PBS and counterstained with 1 mL of DNA staining solution (0.1 mM EDTA, pH 7.4, 50 μg/mL RNase A and 50 μg/mL PI) for 60 min at room temperature. The cells were washed twice with PBS and mounted with 50% glycerol. Stained cells were examined with an Olympus fluorescence upright microscope (Bio-Rad). Apoptotic cells were distinguished easily by typical condensed, fragmented nuclear region from normal cells.

2.6. Measurement of intracellular reactive oxygen species (ROS) formation

An assay based on the conversion of non-fluorescent $DCFH_2$-DA to a fluorescent species by intracellular ROS was used in this study. PC12 cells (per 100 μL in 96-well

plates) were rinsed with Krebs Ringer buffer and loaded with 50 μM $DCFH_2$-DA for 45 min at 37° as per the method of Gunasekar *et al*. [26], and then the cells were washed twice with same buffer and treated with varying concentrations of THP and/or L-DOPA in the presence or absence of antioxidant NAC. After exposure to the compounds, the cells were washed with the Krebs Ringer solution and fluorescence intensity was measured at 475 nm excitation and 525 nm emission.

2.7. ATP assay

After incubation period, the medium was removed and PC12 cells were extracted with lysis buffer. The attached cells scraped from the wells and centrifuged at 12,000 *g* for 15 s. The supernatants were assayed for ATP. The amount of ATP was measured by the luciferase assay system (Promega) using a Microlamat LB 96 P luminometer (EG & G Berthold).

2.8. Statistical analysis

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

3. Results

3.1. Inhibition of cell viability by THP and protection by NAC against THP-induced cytotoxicity

When PC12 cells were treated with 5, 10, 15, and 20 μM of THP, there was a concentration- and time-dependent reduction in the cell viability, which was examined by the MTT assay (Fig. 1). THP at concentrations up to 10 μM did not significantly reduce the cell viability in PC12 cells. However, at concentrations higher than 15 μM, THP caused cytotoxicity. Exposure for 48 hr to THP in PC12 cells was more cytotoxic than exposure for 24 hr.

L-DOPA at concentrations of 20, 50 and 100 μM did not significantly decrease cell viability after 24 hr compared with the untreated control (Fig. 2, upper). However, a significant decrease in cell viability was observed when PC12 cells were exposed to L-DOPA at concentrations higher than 50 μM for 48 hr (Fig. 2, bottom).

In assessing whether THP could stimulate the cytotoxicity of L-DOPA, we added non-cytotoxic or cytotoxic concentrations of THP with L-DOPA to the media. When non-cytotoxic concentrations of THP (5 and 10 μM) were associated with L-DOPA (20, 50 and 100 μM) for 24 or 48 hr, a significant concentration-dependent decrease in cell viability was observed at both incubation time points (Fig. 2). PC12 cells exposure to a cytotoxic concentration of THP (15 μM) in association with L-DOPA (20, 50 and 100 μM) at both incubation time points also resulted in a marked decrease in cell viability compared with L-DOPA alone. Under these conditions, it was noted that THP enhanced the loss of cell viability compared with the untreated control, and that exposure to THP plus L-DOPA for 24 or 48 hr resulted in a marked reduction in the cell viability: exposure for 48 hr was more cytotoxic than that of 24 hr.

The protective effects of NAC were also examined (Fig. 3). A 24- or 48-hr exposure to 0.1 mM NAC alone did not affect cell viability in PC12 cells. THP (15 μM)-, L-DOPA (50 μM)-, and THP (15 μM) plus L-DOPA (50 μM)-induced neurotoxicities were significantly inhibited by the addition of NAC (0.1 mM) to THP- and L-DOPA-containing medium.

3.2. Interaction of THP with L-DOPA-induced apoptosis

To examine whether the cellular damage induced by THP plus L-DOPA might be characteristic of changes leading to apoptosis, we used flow cytometry and the TUNEL technique. In the control PC12 cell cultures for

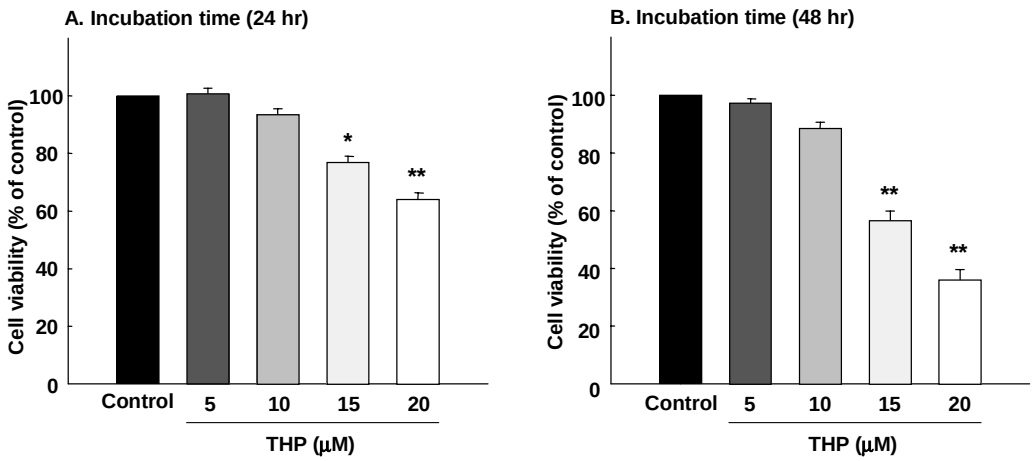

Fig. 1. Effects of THP on PC12 cell viability. PC12 cells were exposed for 24 hr (A) or 48 hr (B) to different concentrations of THP (5, 10, 15 and 20 μM). Cell viability was assessed using MTT methods, in which viable cells convert the soluble dye MTT to insoluble blue formazan crystals. The results represent the means ± SEM of five experiments performed in triplicate. (*) $P < 0.05$, (**) $P < 0.001$ compared with the control (ANOVA followed by Tukey's test).

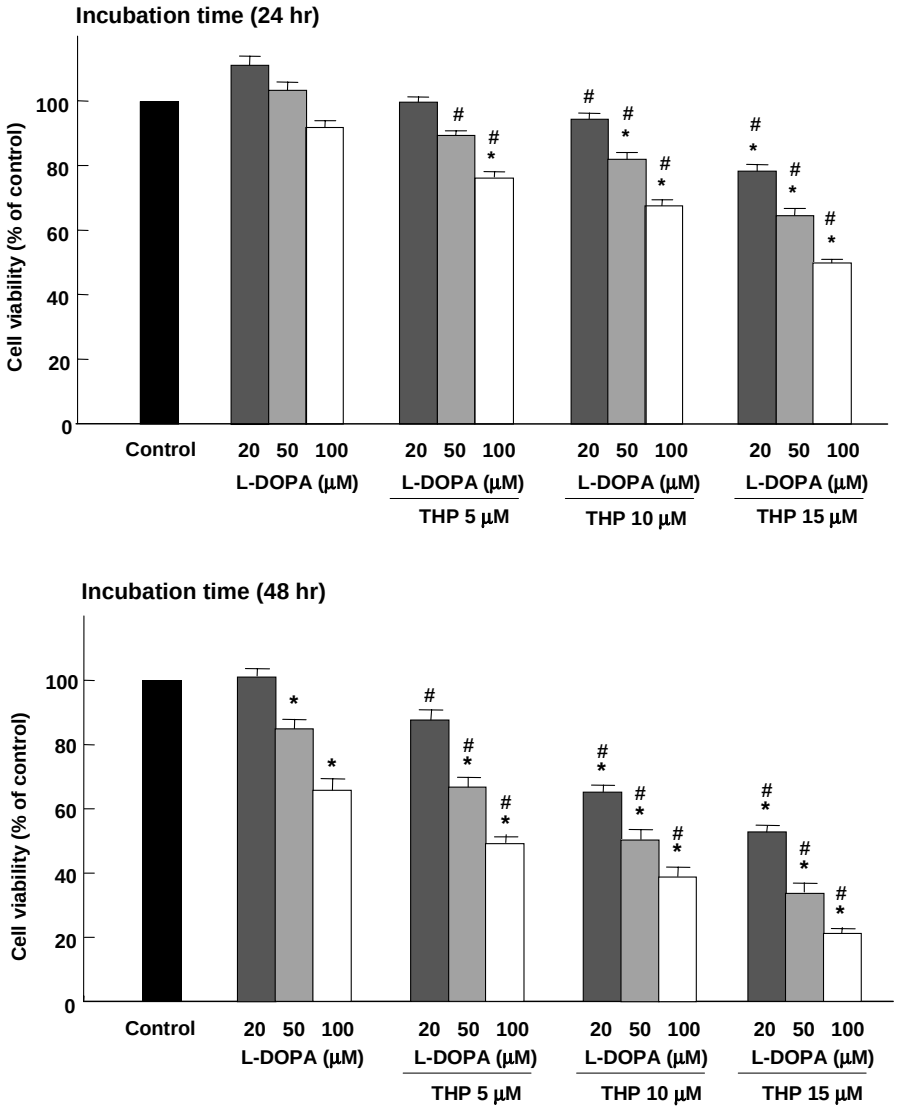

Fig. 2. Enhancing effects of THP on L-DOPA-induced decrease in PC12 cell viability. PC12 cells in culture were incubated in the absence or presence of L-DOPA (20, 50 and 100 μM) associated with THP (5, 10 and 15 μM) for 24 hr (upper) or 48 hr (bottom). Cell viability was assessed using MTT methods, in which viable cells convert the soluble dye, MTT to insoluble blue formazan crystals. The 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).

24 or 48 hr, apoptotic cells were detected neither by flow cytometry (Fig. 4) nor by the TUNEL technique (Fig. 5). Neither THP (5 and 10 μM) nor L-DOPA (20 μM) induced nuclear changes characteristic of apoptosis at both incubation points (data not shown). However, at concentrations higher than 15 μM THP and 50 μM L-DOPA after 48 hr induced apoptotic nuclear changes (Figs. 4 and 5).

When a non-cytotoxic concentration of THP (10 μM) was associated with L-DOPA (20 and 50 μM) for 24 or 48 hr, the percentage of apoptotic cells was markedly increased compared with the cells treated with L-DOPA alone (data not shown). Furthermore, PC12 cells exposure to a cytotoxic concentration of THP (15 μM) in combination with a non-cytotoxic concentration of L-DOPA (20 μM) was more cytotoxic than those with L-DOPA or THP alone. As shown in Fig. 4, a 24-hr (data not shown) or 48-hr exposure to 15 μM THP plus 20 μM L-DOPA revealed 8–11 or 21–24% apoptotic cells percentage, respectively. Exposure to THP (15 μM) added with a cytotoxic concentration of L-DOPA (50 μM) further increased the percentage of apoptotic cells: the percentage of apoptotic cells after a 24-hr (data not shown) or 48-hr exposure was 17–23 or 30–35%, respectively. Exposure of PC12 cells to THP plus L-DOPA for 48 hr markedly increased in the percentage of apoptotic cells compared with exposure for 24 hr. However, NAC (0.1 mM) when added to the cell culture, significantly inhibited both THP- and L-DOPA-induced apoptosis, as revealed by flow cytometry (Fig. 4).

TUNEL technique revealed that treatment with THP (15 μM) plus L-DOPA (20 and 50 μM) for 24 hr (data not shown) and 48 hr (Fig. 5) stimulated cell death via a mechanism, which possessed characteristic morphological

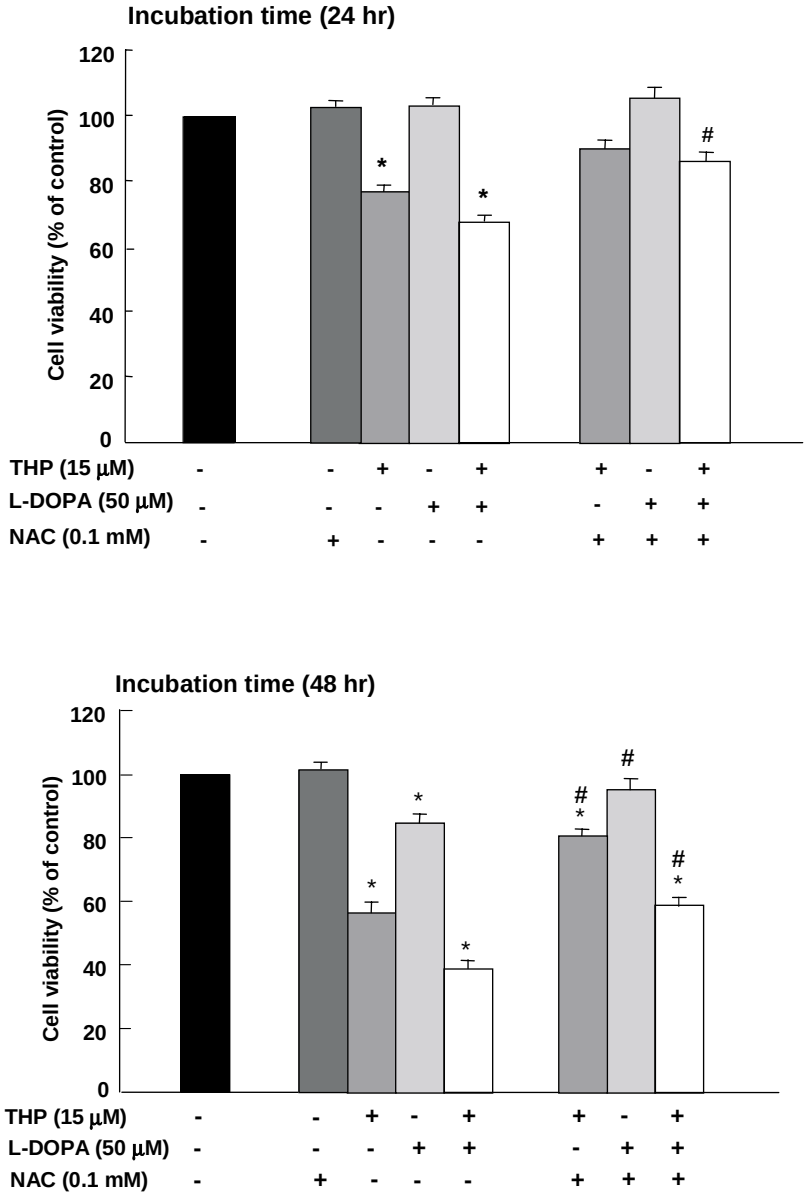

Fig. 3. Effects of NAC on THP plus L-DOPA-induced decrease in PC12 cell viability after 24 hr (upper) or 48 hr (bottom). PC12 cells were cultured in RPMI 1640 medium with 15 μM THP plus 50 μM L-DOPA only or in combination with 0.1 mM NAC. The results represent the means ± SEM of five experiments performed in triplicate. (*), $P < 0.05$ compared with control; (#), $P < 0.05$ compared with corresponding THP + L-DOPA concentrations (ANOVA followed by Tukey's test).

features of apoptotic cell death, including highly condensed chromatin and extensive membrane blebbing. The results have shown that both methods such as flow cytometry and the TUNEL technique produce the similar results for the assessment of apoptotic cells. The enhancing effects of THP on L-DOPA-induced neurotoxicity were also concentration- and time-dependent.

3.3. ROS formation and depletion of intracellular ATP levels by THP

To investigate whether the induction of apoptosis by THP, L-DOPA and their combination was mediated by generation of ROS, the ability of antioxidant to THP and/or L-DOPA-induced apoptosis was examined (Fig. 6). The contribution of THP (5 and 15 μM) and L-DOPA (20 and 50 μM) exposure to the generation of ROS was assayed by loading the cells with $DCFH_2$-DA. A 24-hr (data not shown) or 48-hr exposure of PC12 cells to 15 μM THP markedly increased the generation of ROS. Concurrent exposure of cells to THP (5 and 15 μM) and L-DOPA (20 and 50 μM) produced a significant increase in the intracellular generation of ROS (Fig. 6). THP, L-DOPA and their combination produced a significant increase in intracellular ROS, supporting the involvement of oxidative stress in THP- and L-DOPA-induced apoptosis.

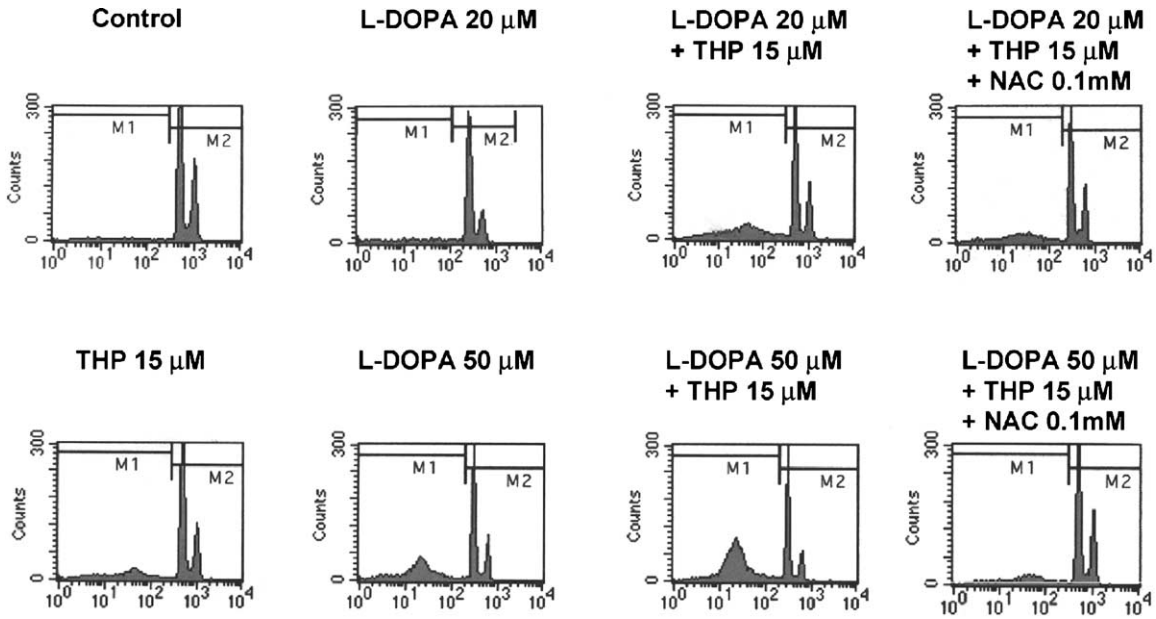

Fig. 4. Flow cytometry histograms of control PC12 cells and PC12 cells after 48 hr exposure to THP (15 μM) alone or associated with L-DOPA (20 and 50 μM). The right histogram refers to PC12 cells after 48 hr exposure to15 μM THP plus 20 or 50 μM L-DOPA associated with 0.1 mM NAC. After incubation, the cells were harvested and stained with PI. DNA relative content was analyzed by flow cytometry. X-axis, DNA content; Y-axis, number of cells.

However, the antioxidant NAC (0.1 mM) significantly inhibited increase in ROS generation induced by THP (5–15 μM) association with L-DOPA (20–50 μM) (Fig. 6).

The intracellular ATP levels were determined to investigate whether the mitochondrial function is significantly impaired by THP and/or L-DOPA. The endogenous ATP levels of PC12 cells incubated 24 or 48 hr with cytotoxic concentrations of THP (15 μM), L-DOPA (50 μM) and their combination were decreased in a rapid and time-dependent manner, while L-DOPA had a slower onset with significant reduction of ATP levels (Fig. 7).

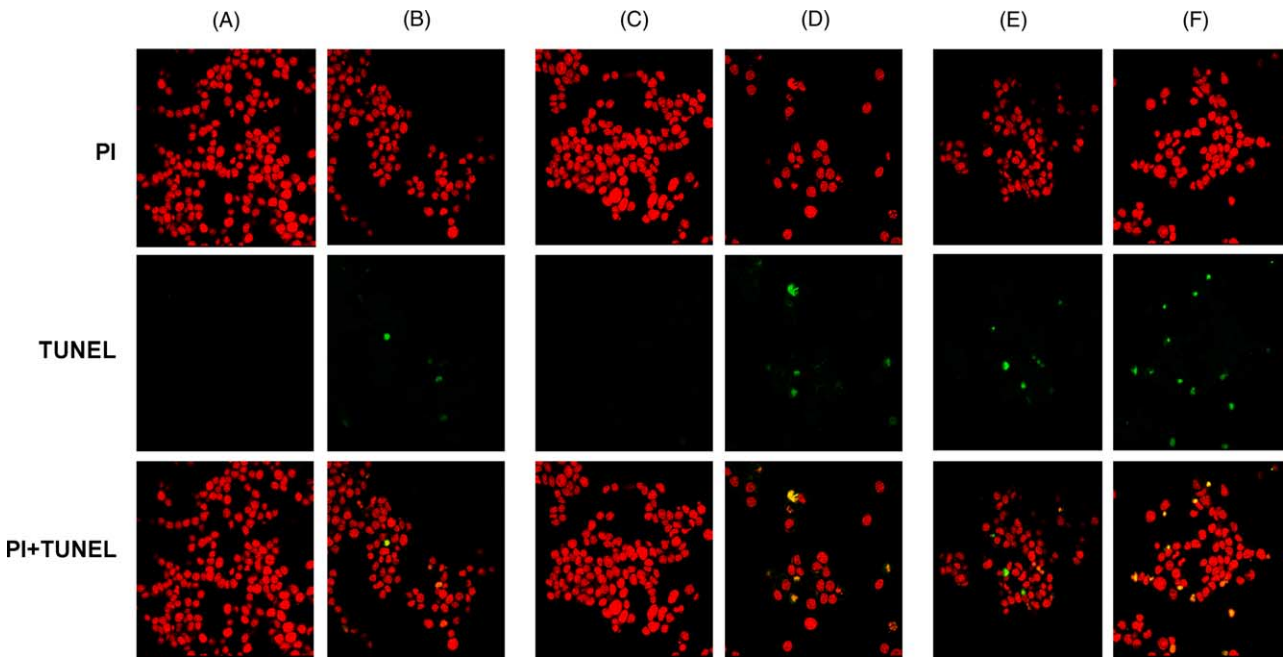

Fig. 5. Enhancing effects of THP on L-DOPA-induced apoptosis in PC12 cells as determined by *in situ* TUNEL. Fluorescence micrographs of untreated PC12 cells (A) and apoptotic PC12 cells (green or yellow green cells) after 48 hr exposure to THP 15 μM (B), L-DOPA 20 μM (C), THP 15 μM + L-DOPA 20 μM (D), L-DOPA 50 μM (E) and THP 15 μM + L-DOPA 50 μM (F). PI was used to counterstain the cells. Apoptotic nuclei are those with green or yellow-green fluorescence.

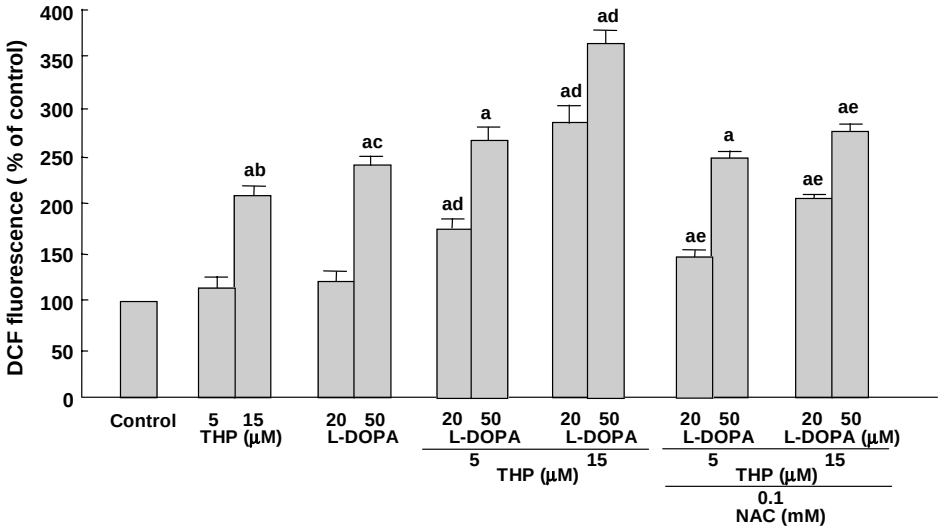

Fig. 6. Effects of NAC on THP- and L-DOPA-induced generation of ROS in PC12 cells loaded with $DCFH_2$-DA. PC12 cells were cultured for 48 hr in RPMI 1640 medium with 5 or 15 μM THP plus 20 or 50 μM L-DOPA only or in combination with 0.1 mM NAC. The formation of peroxides, detected using 2′,7′-dichloroluorescin oxidation and fluorescence, was monitored on a Multi-Well Plate Reader. The results were expressed as a relative percentage of dichlorofluorescin (DCF) fluorescence. Values are the means ± SEM of triplicate determinations in 5–8 distinct experiments. (a) $P < 0.05$ compared with control, (b) $P < 0.05$ compared with 5 μM THP, (c) $P < 0.05$ compared with 20 μM L-DOPA, (d) $P < 0.05$ compared with corresponding L-DOPA concentrations, and (e) $P < 0.05$ compared with corresponding THP + L-DOPA concentrations (ANOVA followed by Tukey's test).

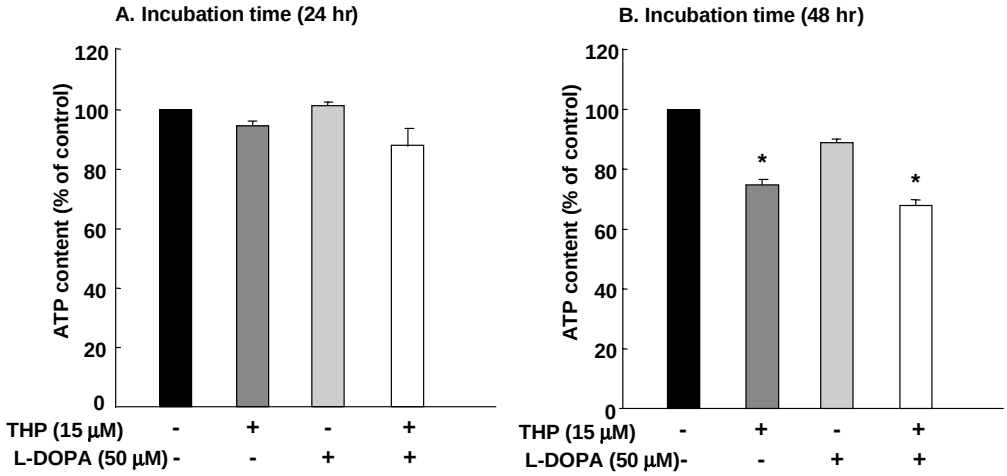

Fig. 7. Effects of THP and L-DOPA on intracellular ATP content in PC12 cells after 24 hr (A) or 48 hr (B). After incubation with toxic concentration of THP (15 μM) and/or L-DOPA (50 μM), PC12 cells were extracted in lysis buffer. The ATP levels were determined by a Microlamat LB 96 P luminometer. The results represent the means ± SEM of five experiments. (*) $P < 0.05$ compared with control (ANOVA followed by Tukey's test).

4. Discussion

Many isoquinoline derivatives are structurally similar to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which causes a Parkinson-like syndrome by forming 1-methyl-4-phenylpyridium (MPP^+) in human and non-human primates [9,27], and there have been reported on the neurotoxicity of isoquinoline compounds [6,28,29]. THP, one of the benzylisoquinoline compounds, has been also linked to Parkinson's disease and brain damage from chronic alcoholism [2,4]. According to our investigation, THP plasma concentrations of Parkinsonian patients receiving L-DOPA therapy (150–200 mg, twice a day) were found with ranges of 0.11–0.34 nM (outpatients from Chungbuk National University Hospital; N = 21), whereas those of healthy volunteers were not detected (data not shown). In addition, THP at concentration ranges of 5–15 μM decreased dopamine content by reducing tyrosine hydroxylase activity in a concentration-dependent manner. L-DOPA at 20–100 μM increased dopamine content in PC12 cells, but the increase in dopamine levels by L-DOPA was in part inhibited when L-DOPA was associated with 5–15 μM THP (data not shown). However, despite THP and its related alkaloids cause dopaminergic neuronal cell death in the experimental models of Parkinsonism [5,30,31], the biochemical neurotoxic effects of THP have not been evaluated.

L-DOPA at concentrations higher than 50 μM led to cell damages, and that the degree of cell damages was

proportioned by the incubation time (Fig. 2). 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 [32]. The neurotoxic action of L-DOPA is its autoxidation into ROS such as hydrogen peroxide, associated with oxygen free radicals and various quinone derivatives [22,23,33–35]. Therefore, in this study, to investigate whether the induction of apoptosis by L-DOPA was mediated by generation of ROS, the ability of antioxidant to inhibit L-DOPA-induced cytotoxicity was examined. In agreement with previous studies, L-DOPA toxicity was inhibited by the antioxidant NAC, suggesting that activation of apoptosis is mediated by oxygen radicals (Fig. 3). Furthermore, the intracellular ROS was increased by L-DOPA (Fig. 6). Taken together, these results thus suggested that L-DOPA may cause neuronal cell death by an oxidative pathway.

Media containing both THP and L-DOPA turned to an orange-brownish color with time in culture, and this color is an indicative of the presence of quinones [22]. The same color change was also observed in the media containing THP and L-DOPA in the present study. In addition, as the color intensity was greatly attenuated by NAC, it is suggested that NAC inhibits both THP and L-DOPA autoxidation. Thus, NAC may act as a direct inhibitor of THP and L-DOPA autoxidation.

Apoptotic cells shrink in size, undergo membrane blebbing in PC12 cells, and exhibit highly condensed chromatin [36]. Both THP (15 μM) and L-DOPA (50 μM) at cytotoxic concentrations exhibited morphological characteristics of apoptosis such as cell shrinkage and membrane blebbing. In addition, concurrent incubation of the cells with THP and L-DOPA produced a greater effect on neurotoxicity over THP or L-DOPA alone in a concentration- and time-dependent (Figs. 4 and 5). These findings also showed that THP aggravated L-DOPA-induced neurotoxicity in PC12 cells.

A possible mechanism of THP-induced apoptosis has been suggested. Seaton *et al*. [37] have shown that isoquinoline and 1,2,3,4-tetrahydroisoquinolines induce the apoptotic cell death in PC12 cells by a mechanism involving generation of free radicals, perhaps secondary to inhibition of the mitochondrial respiratory chain, and apoptosis in these cells was prevented by the antioxidants such as NAC, pyrrolidine dithiocarbamate and dihydrolipoic acid. As similar to this finding, in this study, NAC significantly prevented the decreases in cell viability and apoptosis induced by the THP alone as well as THP associated with L-DOPA (Figs. 3 and 4). Isoquinoline derivatives may impair antioxidant defense mechanism [37]. Free radical production and impaired antioxidant defense may contribute to the mechanism of action of isoquinoline derivatives in causing neurodegeneration. Therefore, the most important site of action with respect to potential neurotoxicity of isoquinoline compounds is the mitochondrial respiratory chain [8,38–41]. McNaught *et al*. [9] have also found that isoquinoline derivatives such as 1,2,3,4-tetrahydroisoquinoline, THP, tetrahydropapaverine, and salsolinol selectively inhibit mitochondrial complex I (NADH-Q reductase) and II (succinate-Q reductase) activities as well as α-ketoglutarate dehydrogenase activity in rat brain mitochondria fragments and dopamine-containing cells. According to Surh [7], THP undergoes redox cycling through its catechol moiety, thereby generating reactive oxygen intermediates, particularly radicals that can mediate DNA damage and cytotoxicity, suggesting that THP exerts cytotoxicity in PC12 cells, possibly by causing oxidative stress. In this study, NAC attenuated THP-induced apoptosis (Fig. 4), indicating the involvement of oxidative species. In addition, THP produced a significant increase in intracellular ROS generation (Fig. 6), supporting the involvement of oxidative stress in THP-induced apoptosis. THP also reduced ATP levels rapidly and markedly in this study (Fig. 7), which is relevant with the previous reports that THP is a most potent inhibitor of mitochondrial respiration chains [8]. Therefore, the hypothesis that oxidative stress may be the underlying mechanism of THP-induced apoptosis is conformed.

This study demonstrates that PC12 cells exhibited apoptosis following L-DOPA exposure, which was enhanced by THP. The mechanisms by which THP enhances L-DOPA toxicity are largely unknown. THP produces an increase in intracellular ROS, which may contribute to the THP-mediated increase in L-DOPA-induced apoptosis. THP has been shown to disrupt mitochodrial respiration chains [8] by inhibiting the activities of mitochondrial complex I and α-ketoglutarate dehydrogenase [9], which may result in an increased generation of ROS [5,7] or depleted ATP levels. Therefore, all these data indicate that the inhibition of mitochondrial function by THP may be taken into account as an additional mechanism in stimulating L-DOPA-induced neurotoxicity.

In conclusion, THP enhanced both apoptotic process and decrease in cell viability induced by L-DOPA. Oxidative stress should be recognized as one of the mechanisms of both THP- and L-DOPA-induced apoptosis. It is, therefore, suggested that Parkinsonian patients on long-term L-DOPA therapy are potentially at risk from the toxic intermediates such as THP, and their plasma or urine level of THP needs to be monitored as therapeutic and diagnostic indices.

Acknowledgments

The authors sincerely thank the financial support of the Research Center for Bioresource and Health, KOSEF.

References

[1] Nagatsu T. Isoquinoline neurotoxins in the brain and Parkinson's disease. Neurosci Res 1997;29:99–111.

[2] Sandler M, Carter SB, Hunter KR, Stern GM. Tetrahydroisoquinoline alkaloids: in vivo metabolites of L-DOPA in man. Nature 1973;241: 439–43.
[3] Turner AJ, Baker KM, Algeri S, Frigerio A, Garattini S. Tetrahydropapaveroline: formation in vivo and in vitro in rat brain. Life Sci 1974;14:2247–57.
[4] Cashaw JL. Tetrahydropapaveroline in brain regions of rats after acute ethanol administration. Alcohol 1993;10:133–8.
[5] Maruyama W, Sango K, Iwasa K, Minami C, Dostert P, Kawai M, Moriyasu M, Naoi M. Dopaminergic neurotoxins, 6,7-dihydroxy-1-(3′,4′-dihydroxybenzyl)-isoquinolines, cause different types of cell death in SH-SY5Y cells: apoptosis was induced by oxidized papaverolines and necrosis by reduced tetrahydropapaverolines. Neurosci Lett 2000;291:89–92.
[6] Storch A, Ott S, Hwang YI, Ortmann R, Hein A, Frenzel S, Matsubara K, Ohta S, Wolf HU, Schwarz J. Selective dopaminergic neurotoxicity of isoquinoline derivatives related to Parkinson's disease: studies using heterologous expression systems of the dopamine transporter. Biochem Pharmacol 2002;7112:1–12.
[7] Surh YJ. Tetrahydropapaveroline, a dopamine-derived isoquinoline alkaloid, undergoes oxidation: implications for DNA damage and neuronal cell death. Eur J Clin Invest 1999;29:650–1.
[8] Morikawa N, Nakagawa HY, Mizuno Y. Effect of dopamine, dimethoxyphenylethamine, papaverine, and related compounds on mitochondrial respiration and complex activity. J Neurochem 1996; 66:1174–81.
[9] McNaught KStP, Carrupt PA, Altmore C, Cellamare S, Carotti A, Testa B, Jenner P, Marsden CD. Isoquinoline derivatives as endogenous neurotoxins in the aetiology of Parkinson's disease. Biochem Pharmacol 1998;56:921–33.
[10] Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 1976;73:2424–8.
[11] Tischler AS, Perlman RL, Morse GM, Sheard BE. Glucocorticoids increase catecholamine synthesis and storage in PC12 pheochromocytoma cell culture. J Neurochem 1983;40:364–70.
[12] Itano Y, Kitamura Y, Nomura Y. 1-Methyl-4-phenylpyridium (MPP^+)-induced cell death in PC12 sells: inhibitory effects of several drugs. Neurochem Int 1994;25:419–24.
[13] Marsden CD. Parkinson's disease. J Neurol Neurosurg Psychiatry 1994;57:672–81.
[14] Lloyd KG, Davidson L, Hornykiewicz O. The neurochemistry of Parkinson's disease: effect of L-DOPA therapy. J Pharmacol Exp Ther 1975;195:453–64.
[15] Hornykiewicz O. The mechanisms of action of L-DOPA in Parkinson's disease. Life Sci 1994;15:1249–59.
[16] Marsden CD, Parkes JD. "On-off" effects in patients with Parkinson's disease on chronic levodopa therapy. Lancet 1976;1:292–6.
[17] Marsden CD, Parkes JD. Success and problems of long-term levodopa therapy in Parkinson's disease. Lancet 1977;1:345–9.
[18] Friedman A, Sienkiewicz J. Psychotic complications of long-term levodopa therapy in Parkinson's disease. Acta Neurol Scand 1991; 84:111–3.
[19] Ogawa N. Levodopa and dopamine agonists in the treatment of Parkinson's disease: advantages and disadvantages. Eur J Neurol 1994;34(Suppl 3):20–8.
[20] Blunt SB, Jenner P, Marsden CD. Suppressive effect of L-DOPA on dopamine cells remaining in the ventral tegmental area of rats previously exposed to the neurotoxin 6-hydroxydopamine. Mov Disord 1993;8:129–37.
[21] Walkinshaw G, Waters CM. Induction of apoptosis in catecholaminergic PC12 cells by L-DOPA. Implications for the treatment of Parkinson's disease. J Clin Invest 1995;95:2458–64.
[22] Basma AN, Morris EJ, Nicklas WJ, Geller HM. L-DOPA cytotoxicity to PC12 cells in culture is via its autoxidation. J Neurochem 1995;64:825–32.
[23] Migheli R, Godani C, Bciola L, Delodu MR, Serra PA, Zangani D, Natale GD, Miele E, Desole MS. Enhancing effect of manganese on L-DOPA-induced apoptosis in PC12 cells: role of oxidative stress. J Neurochem 1999;73:1155–63.
[24] Sandstrom PA, Manhie MD, Buttke TH. Inhibition of activation-induced death in T cell hybridomas by thiol antioxidant: oxidative stress as a mediator of apoptosis. J Neurocyte Biol 1994;55: 221–6.
[25] Mosman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J Immunol Methods 1983;65:55–63.
[26] Gunasekar PG, Kanthasamy AG, Borowitz JL, Isom GE. NMDA receptor activation produces concurrent generation of reactive oxygen species: implication for cell death. J Neurochem 1995;65: 2016–21.
[27] Ohta S. Isoquinolines in Parkinson's disease. In: Storch A, Collins MA, editors. Neurotoxic factors in Parkinson's disease and related disorders, vol. 3. New York: Plenum Press; 2000. p. 33–42.
[28] McNaught KStP, Thull U, Carrupt PA, Altmore C, Cellamare S, Carotti A, Teats B, Jenner P, Marsden CD. Toxicity to PC12 cells of isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurosci Lett 1996;206:37–40.
[29] Bai J, Nakamura H, Hattori I, Tanito M, Yodoi J. Thioredoxin suppresses 1-methyl-4-phenylpyridinium-induced neurotoxicity in rat PC12 cells. Neurosci Lett 2002;321:81–4.
[30] Langston JW, Irwin I. MPTP: current concepts and controversies. Clin Neuropharmacol 1986;9:485–507.
[31] Seyfried J, Soldner F, Jung WS, Schulz JB, Klockgether T, Kovar KA, Wullner U. Effect of 1-methyl-4-phenylpyridinium on glutathione in rat pheochromocytoma PC12 cells. Neurochem Int 2000;35: 489–97.
[32] Rossor MN, Watkins J, Brown MJ, Reid JL, Dollery CT. Plasma levodopa dopamine and therapeutic response following levodopa therapy of Parkinsonian patients. Brain Res 1980;46:385–92.
[33] Rosenberg PA. Catecholamine toxicity in cerebral cortex in dissociated cell culture. J Neurosci 1988;8:2887–94.
[34] Walkinshaw G, Waters CM. Induction of apoptosis in catecholaminergic PC12 cells by L-DOPA. Implications for the treatment of Parkinson's disease. J Clin Invest 1995;95:2458–64.
[35] Smith TS, Parker WD, Bennett JP. L-DOPA increases nigral production of hydroxyl radicals in vivo: potential L-DOPA toxicity? Neuroreport 1994;5:1009–11.
[36] Vermes I, Haanen C. Apoptosis and programmed cell death in health and disease. Adv Clin Chem 1994;31:177–246.
[37] Seaton TA, Cooper JM, Schapira AHV. Free radical scavengers protect dopaminergic cell lines from apoptosis induced by complex inhibitors. Brain Res 1997;777:110–8.
[38] McNaught KStP, Thull U, Carrupt PA, Altmore C, Cellamare S, Carotti A, Testa B, Jenner P, Marsden CD. Inhibition of complex I by isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Biochem Pharmacol 1995;50: 1903–11.
[39] McNaught KStP, Altmore C, Cellamare S, Carotti A, Thull U, Carrupt PA, Testa B, Jenner P, Marsden CD. Inhibition of α-ketoglutarate dehydrogenase by isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Neuroreport 1995;6:1105–8.
[40] McNaught KStP, Thull U, Carrupt PA, Altmore C, Cellamare S, Carotti A, Testa B, Jenner P, Marsden CD. Effects of isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on mitochondrial respiration. Biochem Pharmacol 1996;51:1503–11.
[41] Morikawa N, Naoi N, Maruyama W, Ohta S, Kotake Y, Kawai H, Niwa T, Dostert P, Mizuno Y. Effects of various tetrahydroisoquinoline derivatives on mitochondrial respiration and electron transfer complexes. J Neural Trans 1998;105:677–88.