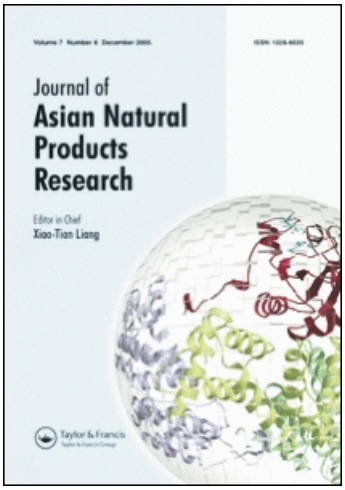

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Catalponol enhances dopamine biosynthesis and protects against L-DOPA-induced cytotoxicity in PC12 cells

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The effects of catalponol (**1**) on dopamine biosynthesis and L-DOPA-induced cytotoxicity in PC12 cells were investigated. Catalponol at concentration ranges of 1–5 μM increased the intracellular levels of dopamine at 12–48 h. Catalponol at concentrations of up to 10 μM did not alter cell viability. Tyrosine hydroxylase (TH) activity was enhanced by **1** at 3 μM in a time-dependent manner, but aromatic L-amino acid decarboxylase activity was not. Catalponol also increased the intracellular levels of cyclic AMP and TH phosphorylation. In addition, catalponol at 3 μM associated with L-DOPA (20–50 μM) further enhanced the increases in dopamine levels induced by L-DOPA (50–100 μM) at 24 h. Catalponol at 2–5 μM inhibited L-DOPA (100–200 μM)-induced cytotoxicity at 48 h. These results suggest that **1** enhanced dopamine biosynthesis by inducing TH activity and protected against L-DOPA-induced cytotoxicity in PC12 cells, which was mediated by the increased levels of cyclic AMP.

Keywords: catalponol; dopamine biosynthesis; cyclic AMP; tyrosine hydroxylase; L-DOPA-induced cytotoxicity; PC12 cells

1. Introduction

Catalpa ovata G. Don (Bignoniaceae) is widely grown in Korea and its fruit has been used as a diuretic for chronic nephritis [1]. The root and stem bark of *C. ovata* are also used for treating fever, jaundice, and edema, which are caused by nephritis [2]. *C. ovata* has been found to have several bioactive compounds such as naphthoquinone derivatives [2,3], flavone glycosides [4], and monoterpene glycosides [5].

As part of our ongoing research to investigate the effects on dopamine biosynthesis and 3,4-L-dihydroxyphenylalanine (L-DOPA)-induced cytotoxicity from natural products, it has been found that a methanol (MeOH) extract from the stem bark of *C. ovata* increased the intracellular levels of dopamine in PC12 cells. The MeOH extract was therefore subjected to the bioactivity-guided fractionations to isolate the bioactive components and the final purified bioactive substance was identified as a naphthoquinone congener, i.e. catalponol (**1**) [3,6] (Figure 1). Naphthoquinone derivatives from *C. ovata* display various pharmacological functions including anti-bacterial, anti-fungal, and anti-tumor activities [2].

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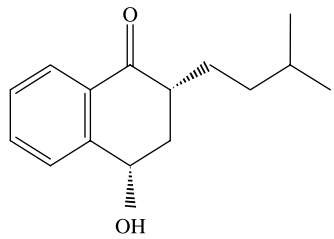

Figure 1. Chemical structure of catalponol (**1**).

In dopamine biosynthetic pathways, L-DOPA is formed from L-tyrosine by tyrosine hydroxylase (EC 1.14.16.2, TH), a rate-limiting enzyme, and is then converted to dopamine by aromatic L-amino acid decarboxylase (EC 4.1.1.28, AADC). L-DOPA, as the precursor of dopamine, is the most frequently prescribed drug for controlling the symptoms of Parkinson's disease [7]. However, L-DOPA may accelerate the deterioration of the parkinsonian patients. L-DOPA is toxic toward cultured sympathetic and striatal neurons, and PC12 cells [8,9] due to auto-oxidation and enzymatic oxidation by generating reactive oxygen species (ROS) leading to apoptosis.

The intracellular levels of cyclic AMP are known to regulate TH activity and TH gene expression via protein kinase A (PKA) and protein kinase C in PC12 cells, respectively [10]. Cyclic AMP also exerts protective effects against oxidative stress-induced apoptotic cell death in PC12 cells [11].

PC12, rat adrenal pheochromocytoma, cell lines have dopaminergic neuronal properties and also express dopamine biosynthetic enzymes such as TH and AADC [12]. PC12 cells have therefore been used as a model system for investigating the effects on dopamine biosynthesis, neurocytotoxicity, signal transduction, and neuronal differentiation [13].

In the present study, the effects of **1** on dopamine biosynthesis and L-DOPA-induced cytotoxicity in PC12 cells were investigated in order to confirm the further pharmacological functions.

2. Results and discussion

2.1 Intracellular levels of dopamine

Treatment of PC12 cells with **1** at concentration ranges of 1–5 μM significantly increased the intracellular levels of dopamine from 110 to 145% for 48 h in PC12 cells (Table 1). Catalponol at 3 μM showed the maximal levels for 48 h. The intracellular levels of dopamine were also enhanced by **1** in a time-dependent manner for 12–48 h (Table 2). Catalponol did not affect cell viability at concentrations up to 10 μM, which was determined by an MTT assay (data not shown). The concentration of 3 μM catalponol was therefore chosen for the following experiments. In addition, dopamine levels in the medium,

Table 1. Effects of **1** on the intracellular levels of dopamine in PC12 cells.

Catalponol concentration (μM)	Dopamine levels (% of control)
Control (0)	3.41 ± 0.36 (100)
1.0	4.02 ± 0.28 (118)*
2.0	4.81 ± 0.37 (141)**
5.0	4.60 ± 0.31 (135)*
7.0	3.95 ± 0.24 (116)*

Notes: The control values of the intracellular dopamine levels were 3.41 ± 0.36 nmol/mg protein. Results represent means ± SEM of four experiments. Significantly different from control values: *$p < 0.05$, **$p < 0.01$ (ANOVA followed by Tukey's test).

Table 2. Effects of **1** on the time courses of dopamine levels in PC12 cells.

Incubation times (h)	Dopamine levels (% of control)
Control (0)	3.44 ± 0.34 (100)
12	3.96 ± 0.25 (115)*
24	4.20 ± 0.38 (122)*
36	4.39 ± 0.35 (128)*
48	5.26 ± 0.41 (153)**

Notes: The control values of the intracellular dopamine levels were 3.44 ± 0.34 nmol/mg protein. For further comments, see Table 1.

which was secreted by the intracellular dopamine, were slightly increased by 3 μM catalponol for 3–48 h, but the increase was not significant (data not shown).

2.2 TH and AADC activities and TH phosphorylation

As shown in Table 3, catalponol at 3 μM increased the intracellular activity of TH for 24–48 h. TH activity reached the maximal level of 131.4% for 36–48 h. However, AADC activity was not altered by **1** (data not shown). When the cells were replaced with a fresh medium after exposure to 3 μM catalponol at 48 h, TH activity was recovered to *c.* 95–104% of control levels at 72 h (data not shown). TH phosphorylation (Ser 40) was also increased by *c.* 1.5- to 2.0-fold when compared to that of control levels for 24–48 h by 3 μM catalponol (Figure 2).

2.3 Intracellular levels of cyclic AMP

At a concentration of 3 μM, catalponol increased the intracellular levels of cyclic AMP to 108–124% when compared to that of control levels at 20–60 min (control levels of cyclic AMP, 235.7 ± 13.8 pmol/mg protein; Table 4).

2.4 L-DOPA-induced dopamine biosynthesis

L-DOPA at 50–100 μM increased the intracellular levels of dopamine to 242–326 and 124–139%, respectively, for 24 and 48 h compared to L-DOPA-untreated controls in PC12 cells (Figure 3). Catalponol at 3 μM associated with L-DOPA (50 and 100 μM) for 24 or 48 h enhanced L-DOPA-induced increases in dopamine levels compared to L-DOPA alone (Figure 3).

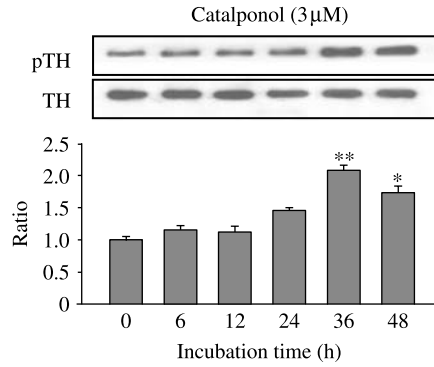

Figure 2. Effects of **1** on TH phosphorylation illustrated on representative blots of TH and phosphor-TH. PC12 cells were treated with catalponol (3 μM) and immunoblots of lysates from treated cells were probed with phosphor-TH (upper panel) and TH (lower panel) antibodies. The relative density ratio in control levels was expressed as 1 arbitrary unit. Values were means ± SEM ($n = 3$). Significantly different from control values: $*p < 0.05$; $**p < 0.01$ (ANOVA followed by Tukey's test).

Table 3. Effects of **1** on TH activity in PC12 cells.

Incubation times (h)	TH activity (% of control)
Control (0)	3.75 ± 0.26 (100)
12	3.94 ± 0.28 (105)
24	4.16 ± 0.41 (111)*
36	4.61 ± 0.38 (123)*
48	5.06 ± 0.46 (153)**

Notes: The control values of TH activity were 3.75 ± 0.26 nmol/mg protein. For further comments, see Table 1.

Table 4. Effects of **1** on cyclic AMP levels in PC12 cells.

Incubation times (min)	TH activity (% of control)
Control (0)	235.7 ± 13.8 (100)
20	252.2 ± 15.4 (107)
60	293.6 ± 20.1 (125)*

Notes: PC12 cells were treated with catalponol (3 μM) and incubated at 37°C for 20 and 60 min. The intracellular cyclic AMP levels were measured by an enzyme immunoassay system. Cyclic AMP levels of the control values were 235.7 ± 13.8 pmol/mg protein. Values were means ± SEM of four experiments. Significantly different from control values: $*p < 0.05$ (ANOVA followed by Tukey's test).

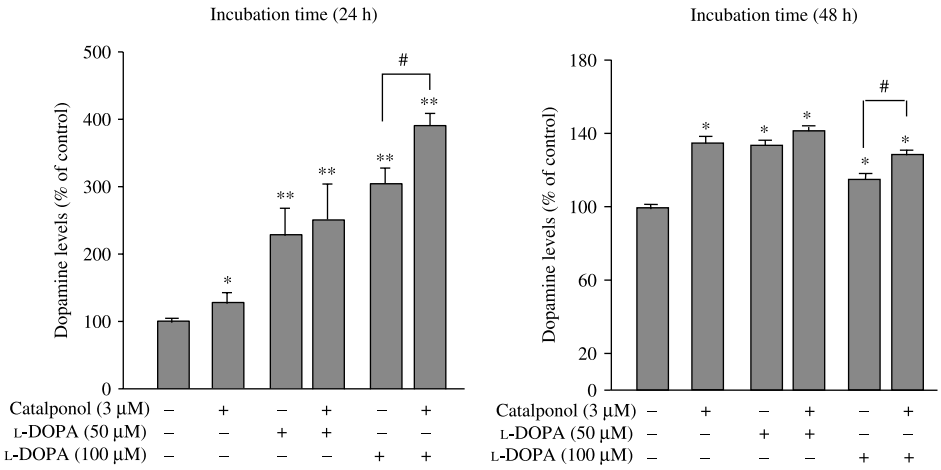

Figure 3. Effects of **1** on L-DOPA-induced dopamine levels in PC12 cells. PC12 cells were exposed to L-DOPA (50 and 100 μM) in the absence or presence of catalponol (3 μM) for 24 and 48 h. The control values of the intracellular dopamine levels were 3.85 ± 0.27 nmol/mg protein. Results represent means ± SEM of four experiments. $*p < 0.05$, $**p < 0.01$ compared to control values; $^{\#}p < 0.05$ compared to the corresponding L-DOPA concentrations (ANOVA followed by Tukey's test).

2.5 *L-DOPA-induced cytotoxicity*

Catalponol- or L-DOPA-untreated cells showed apoptotic cell death below 10% at 24 or 48 h. Catalponol at 3 μM also did not show apoptotic cell death at 24 or 48 h. L-DOPA at 50 and 100 μM reduced cell viability to 79.8 and 70.6%, respectively, for 48 h (Figure 4). However, L-DOPA (50–100 μM)-induced cytotoxicity was significantly reduced by co-treatment

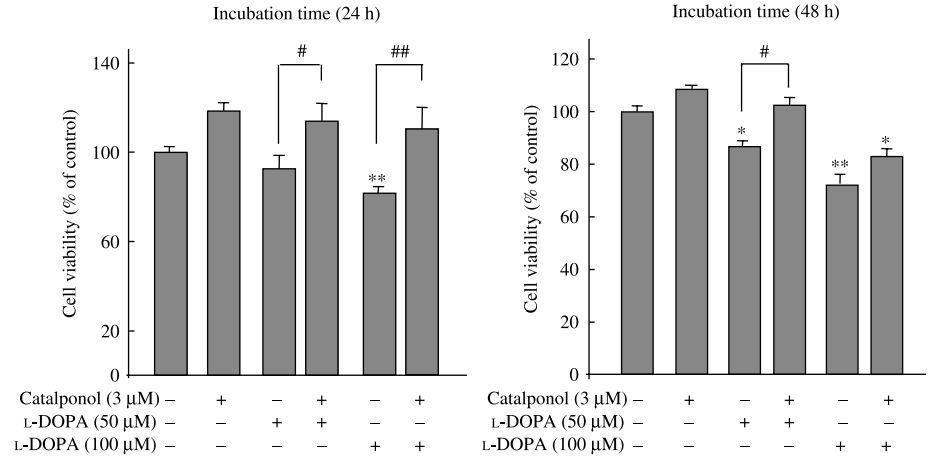

Figure 4. Effects of **1** on L-DOPA-induced cell viability in PC12 cells. PC12 cells were exposed to L-DOPA (50 and 100 μM) in the absence or presence of catalponol (3 μM) for 24 and 48 h and the cell viability was assessed by the conversion of the MTT assay to insoluble blue formazan crystals. Results represent means ± SEM of four experiments. $*p < 0.05$, $**p < 0.01$ compared to control values; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to the corresponding L-DOPA concentrations (ANOVA followed by Tukey's test).

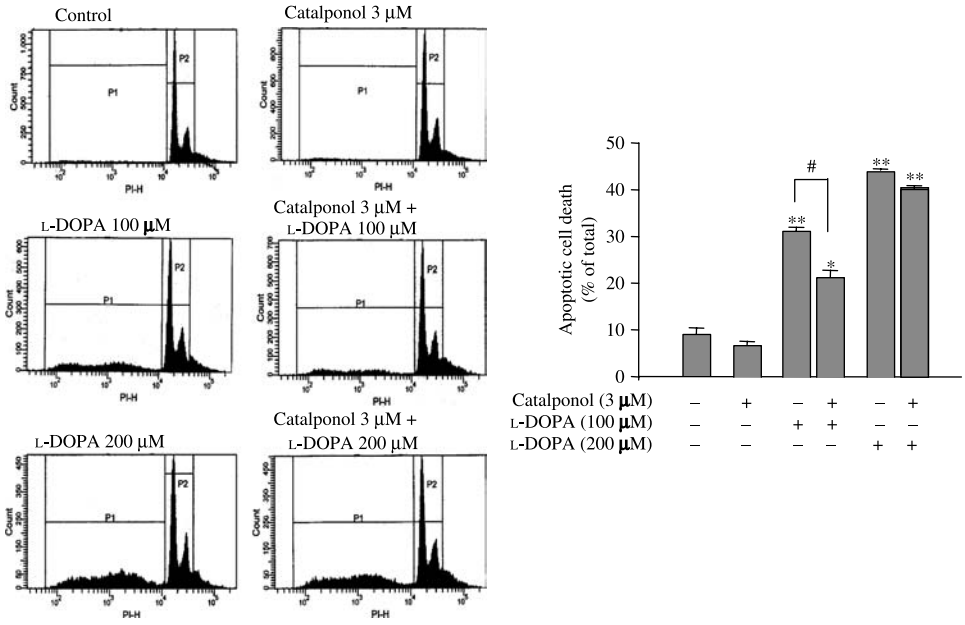

Figure 5. Flow cytometry histograms of control PC12 cells and PC12 cells after 48 h exposure to catalponol (3 μM) alone or associated with L-DOPA (100 and 200 μM). After incubation, the cells were harvested and stained with propidium iodide. DNA relative content was analyzed by flow cytometry (*x*-axis, DNA content; *y*-axis, number of cells). The percentages of apoptotic cells after 48 h exposure were determined by flow cytometry. The results were expressed as means ± SEM of four experiments. $*p < 0.05$, $**p < 0.01$ compared to control levels; $^{\#}p < 0.05$ compared to the corresponding L-DOPA concentrations (ANOVA followed by Tukey's test).

with **1** (3 μM) at 24–48 h (Figure 4). The apoptotic cell death that was induced by 100–200 μM L-DOPA was 32–45% for 48 h compared to L-DOPA-untreated cells (Figure 5). Catalponol (3 μM) associated with L-DOPA (100 and 200 μM) for 48 h reduced the apoptotic cell death, which was induced by L-DOPA alone, suggesting that **1** showed the protective activity against L-DOPA-induced cytotoxicity (Figure 5). The characteristic morphological features of apoptotic cell death were also observed, including highly condensed chromatin and extensive membrane blebbing (data not shown).

2.6 Discussion

Quinone and isoquinoline derivatives induce cytotoxicity due to the formation of ROS mediated by their redox cycling in neuronal and cultured cells [14,15]. In contrast, an *o*-naphthoquinone derivative, biflorin, shows anti-apoptotic and anti-oxidative activities [16]. In this study, the effects of **1** (Figure 1), a naphthoquinone derivative, on dopamine content and L-DOPA-induced cytotoxicity in PC12 cells were investigated.

Catalponol significantly increased the intracellular levels of cyclic AMP. Compound **1** also induced TH activity and TH phosphorylation, but not AADC activity (Tables 3 and 4). Intracellular cyclic AMP is known to activate TH activity and TH gene expression via PKA in neuronal and PC12 cells [10]. These results suggested that **1** showed an inducing effect on dopamine biosynthesis by enhancing TH activity and TH phosphorylation mediated by cyclic AMP in PC12 cells.

Cyclic AMP regulates intracellular growth, survival, and proliferation primarily by modulating extracellular signal-regulated kinase [17]. The increased levels of intracellular cyclic AMP exert a protective activity against oxidative stress-induced apoptotic cell death in PC12 cells [11]. Dibutyryl cyclic AMP, as an analog of cyclic AMP, also protects against ROS formation [11].

L-DOPA at low concentrations (3–30 μM) protects PC12 cells from cytotoxicity by activating mitogen-activated protein kinase [18]. In contrast, L-DOPA at concentrations higher than 50 μM produces oxidative stress-induced cytotoxicity by the formation of ROS such as hydrogen peroxide and quinone derivatives toward neuronal and PC12 cells [9,19]. L-DOPA at 20–200 μM in a cultured medium of PC12 cells increases the intracellular levels of dopamine after 24 and 48 h [20]. L-DOPA at 20–50 μM has shown a greater increase in dopamine levels than L-DOPA at 100–200 μM, which is due to the L-DOPA-induced oxidative stress [21].

Catalponol (3 μM) associated with L-DOPA (50–100 μM) further enhanced L-DOPA-induced increases in dopamine levels (Figure 3) in PC12 cells and also protected against L-DOPA (50–100 μM)-induced cytotoxicity by reducing the apoptotic cell death (Figures 4 and 5). It is therefore suggested that the increased cyclic AMP levels by **1** play an important role in the protection against L-DOPA-induced cytotoxicity. It is also proposed that L-DOPA (20–200 μM) increased the intracellular levels of cyclic AMP to protect against L-DOPA-induced cytotoxicity in PC12 cells [21].

Many bioactive components from natural products are reported to have a modulatory effect on dopamine biosynthesis in PC12 cells. Isoquinoline derivatives such as bulbocapnine, berberine, palmatine, and hydrastine had been found to lower the dopamine content by reducing the TH activity in PC12 cells [22,23]. In addition, dihydro- and tetrahydro-isoquinolines, salsolinol, norsalsolinol, papaverine, tetrahydropapaverine, and tetrahydropapaveroline have neurotoxic effects against neuronal and PC12 cells [14,15]. Hydrastine is grouped into a phthalide-isoquinoline alkaloid [23]. Catalpalactone, which has a phthalide and quinone ring, inhibits dopamine biosynthesis by reducing TH and AADC activities and enhances L-DOPA-induced cytotoxicity [24]. These results suggest that phthalide and quinones or isoquinoline derivatives might be able to inhibit dopamine biosynthesis in PC12 cells. In contrast, scoparone, a coumarin derivative, shows an increasing effect on dopamine biosynthesis and protects against L-DOPA-induced cytotoxicity in PC12 cells [25].

In conclusion, **1** enhanced dopamine biosynthesis by inducing TH activity and also showed a protective effect on L-DOPA-induced cytotoxicity in PC12 cells. In addition, *C. ovata* proved to have two kinds of compounds, catalponol and catalpalactone [24], which have an opposite function for dopamine biosynthesis and L-DOPA-induced cytotoxicity in PC12 cells. Therefore, the MeOH extract of the stem bark from *C. ovata* may need to be rechecked for pharmacological functions according to the ratio of the bioactive components including catalponol and catalpalactone.

3. Experimental

3.1 Chemicals

L-DOPA, dopamine, L-tyrosine, DL-6-methyl-5,6,7,8-tetrahydropterine, catalase, 3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2*H*-tetrazolium bromide (MTT), and ribonuclease A were purchased from Sigma Chemical Co. (St Louis, MO, USA). A cyclic AMP enzyme immunoassay system kit was purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, England). Primary antibodies against TH, phosphor-

TH, and anti-rabbit HRP-linked lgG were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). All sera, antibiotics, and RPMI 1640 for cell culture were obtained from Gibco (Grand Island, NY, USA). All other chemicals were of reagent grade.

3.2 Separation and identification of catalponol

The stem bark of *C. ovata* was collected at the herb garden at Chungbuk National University (Cheongju, Korea). A voucher specimen (No. CBNU0401) was deposited at the Herbarium of the College of Pharmacy, Chungbuk National University. The dried stem bark of *C. ovata* (1.3 kg) was extracted with 100% MeOH. The dried MeOH extract (74.8 g) was then partitioned in turn with dichloromethane (CH_2Cl_2), ethylacetate (EtOAC), and water. The CH_2Cl_2 extract (10.5 g) was subjected to silica gel chromatography (5 × 25 cm, 70–230 mesh) using gradient mixtures with CH_2Cl_2:MeOH (80:1, 50:1, 20:1, 10:1, 5:1 v/v) to obtain subfractions (CO-A–CO-E). Fractions of CO-B1 to CO-B3 from CO-B fraction were separated by silica gel column chromatography (hexane–EtOAc gradient; 4 × 21 cm; 70–230 mesh). A bioactive component, which induced dopamine biosynthesis, was isolated from CO-B2 fraction and identified as a naphthoquinone derivative, catalponol (**1**) (10.9 mg: purity, 98.7%; Figure 1): colorless powder; mp 133–137°C; $[\alpha]_D^{25}$=11.0 (c = 1.2, MeOH); EI-MS *m/z*: 230 $[M]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz): 2.50 (2-H), 1.78, 2.56 (3-H), 5.01 (4-H), 8.03 (5-H), 7.40 (6-H), 7.60 (7-H), 7.73 (8-H), 2.30, 2.73 (11-H), 5.16 (12-H), 1.72 (14-H), 1.65 (15-H); ^{13}C NMR ($CDCl_3$, 125 MHz): 199.3 (1-C), 47.32 (2-C), 39.41 (3-C), 69.19 (4-C), 127.89 (5-C), 134.40 (6-C), 128.51 (7-C), 126.37 (8-C), 147.17 (9-C), 131.89 (10-C), 28.66 (11-C), 121.94 (12-C), 134.71 (13-C), 26.54 (14-C), 18.56 (15-C). ^{1}H and ^{13}C NMR spectra and physical constants were identical to those obtained in the previous reports [3]. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-500 MHz NMR spectrometer (Rheinstetten, Germany). EI-MS was recorded on a Hewlett Packard 598A mass spectrometer (Palo Alto, CA, USA).

3.3 Cell culture

PC12 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2/humidified air [12].

3.4 Determination of dopamine levels

Dopamine content was determined by an HPLC method [25,26]. The pellet extract with trichloroacetic acid (1 M, 100 μl) and isoproterenol (200 pmol, internal standard) was passed through a Toyopak SP-M cartridge (Na^+, resin 1 ml; Toso, Tokyo, Japan). The adsorbed amines were eluted with 0.6 M KCl–acetonitrile (1:1, v/v, 2 ml), and then the cartridge eluates were derivatized with 1,2-diphenylethylenediamine. The final reaction mixtures were injected into an HPLC system (Toso).

3.5 Assay for TH and AADC activities

TH activity was measured according to a slightly modified procedure of Nagatsu *et al.* [27] as described previously [23]. L-DOPA, which was produced by an enzyme reaction (8 min) from the substrate L-tyrosine, was determined using an HPLC equipped with an electrochemical detector (CM8010, Toso). AADC activity was also measured by the method of Yin *et al.* [23]. The amount of dopamine formed by an enzymatic reaction from the substrate L-DOPA was determined by an HPLC system (Toso) [23].

3.6 Assay for TH phosphorylation: western blot analysis

TH phosphorylation was analyzed by western blot analysis [28]. Protein samples (30 μg) were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride blots at 300 mA for 3 h. After incubating with primary and secondary antibodies, the transferred proteins were incubated with ECL substrate solution (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) for 1 min according to the manufacturer's instructions, and visualized using a radiographic film.

3.7 Determination of cyclic AMP levels

The intracellular levels of cyclic AMP were measured using an enzyme immunoassay system kit. PC12 cells were incubated overnight in a 96-well plate at 37°C. The cells were incubated for a suitable time and agitated after the lysis reagent was added. Finally, the absorbance was determined at 450 nm using a Bauty Diagnostic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

3.8 Assessment of cell viability

Cell viability was determined by the conventional MTT assay with slight modification [29]. The MTT solution was added to each well and incubated at 37°C for 3–4 h. Then, the reaction was stopped by adding 0.8 M HCl in isopropanol through mixing. The absorbance was measured at 570 nm using a microplate reader (Molecular Devices).

3.9 Flow cytometric analysis of apoptotic cells

PC12 cells were fixed in ice-cold 70% ethanol and suspended in 1 ml staining solution (50 μg/ml propidium iodide, 0.05 mg/ml ribonuclease A, and 0.1 mM EDTA, pH 7.4). The cellular DNA content was analyzed using a FACScan flow cytometer and the diagrams were recorded using a FACS vantage fluorescence-activated flow cytometer (Becton Dickinson, San Jose, CA, USA). The calculation of the percentage of apoptotic cells was based on the cumulative frequency curves of the appropriate DNA histograms.

3.10 Statistical analysis

Protein content was determined using bovine serum albumin as a standard [30]. All data were presented as means ± SEM of at least four experiments. Statistical analysis was performed using ANOVA followed by Tukey's test.

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