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Effects of asimilobine on dopamine biosynthesis and L-DOPA-induced cytotoxicity in PC12 cells

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The effects of asimilobine, an aporphine isoquinoline alkaloid, on dopamine biosynthesis and L-DOPA-induced cytotoxicity in PC12 cells were investigated. Asimilobine at concentration ranges of 0.05–0.2 μM showed a significant inhibition of intracellular dopamine levels for 24 h in a concentration-dependent manner with an IC_{50} value of 0.13 μM . Asimilobine at 0.15 μM inhibited tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AADC) activities at 24 h (73.2% inhibition of TH activity): the inhibition of TH activity was stronger and longer than that of AADC activity. Asimilobine also decreased TH mRNA levels and intracellular cyclic AMP levels, but not the basal Ca^{2+} concentrations. In addition, asimilobine at 0.05–5.0 μM , but not 10 μM , did not alter cell viability toward PC12 cells. A non-cytotoxic asimilobine (0.15 μM) associated with L-DOPA (20, 50, and 100 μM) for 24 h inhibited L-DOPA-induced increases in dopamine levels and enhanced L-DOPA-induced cell death when compared with L-DOPA alone. These results suggest that asimilobine inhibits dopamine biosynthesis by mainly reducing the TH activity and TH mRNA expression, and enhances L-DOPA-induced cytotoxicity in PC12 cells.

Keywords: asimilobine; dopamine biosynthesis; tyrosine hydroxylase; L-DOPA-induced cytotoxicity; PC12 cells

1. Introduction

Asimilobine, which belongs to an aporphine isoquinoline alkaloid (Figure 1), has been isolated from plant species of *Magnolia obobata* Thun. (Magnoliaceae). The stem barks of *M. obobata* have been applied frequently for the treatment of stomachic disorders in traditional oriental medicines. Asimilobine shows an antimalarial activity [1] and is also responsible for the cytotoxicity against human tumor cell lines [2].

In dopamine biosynthetic pathways, dopamine is directly biosynthesized by

tyrosine hydroxylase (TH, EC 1.14.16.2) and aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28). TH is the rate-limiting enzyme and catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) [3], which is, then, converted to dopamine by AADC [4]. The TH activity can be regulated by the phosphorylation through the control of protein kinase A, protein kinase C, and Ca^{2+} /calmodulin kinase II [5,6], Intracellular cyclic AMP levels and Ca^{2+} concentrations are also known to regulate TH activity and TH gene expression via protein

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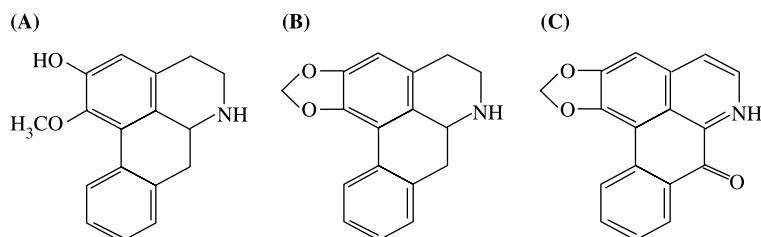


Figure 1. Chemical structures of asimilobine (1), anonaine (2), and liriodenine (3).

kinase A and protein kinase C in neuronal and PC12 cells [6–7]. L-DOPA is frequently prescribed for the therapy of Parkinson's disease [8]. However, the L-DOPA long-term therapy induces the dopaminergic cell damages in Parkinsonian patients [9,10]. L-DOPA also induces oxidative stress-mediated apoptosis by producing reactive oxygen species (ROS) in neuronal and PC12 cells [9–12].

Isoquinoline alkaloids are formed by the condensation of tyrosine and/or L-DOPA and have various dopamine-related pharmacological activities. Among them, berberine, palmatine, bulbocapnine, higenamine, tetrahydropapaveroline, ethaverine, and hydrastine have the inhibitory effects on dopamine biosynthesis in PC12 cells [13–18]. Berberine, palmatine, and hydrastine competitively inhibit bovine adrenal TH [13,17], and tetrahydropapaveroline and

bulbocapnine uncompetitively does it [18,19]. Tetrahydropapaveroline and hydrastine inhibit L-DOPA-induced increases in the dopamine content and aggravate L-DOPA-induced cytotoxicity in PC12 cells [12,17,20].

Aporphine isoquinoline alkaloids such as bulbocapnine, liriodenine, and anonaine show the inhibitory effects on dopamine biosynthesis in PC12 cells [14,21]. Bulbocapnine has antiperoxidative and dopamine D₁ receptor antagonistic effects [22]. Boldine, glaucine, and some halogenated boldine derivatives, which also belong to aporphine alkaloids, appear to exhibit antioxidant and anti-inflammatory properties [23,24], which are based on the aporphine skeleton [25], and brain anti-dopaminergic activities [26,27]. These results suggest that the aporphine isoquinoline derivatives might be able to modulate dopamine biosynthesis.

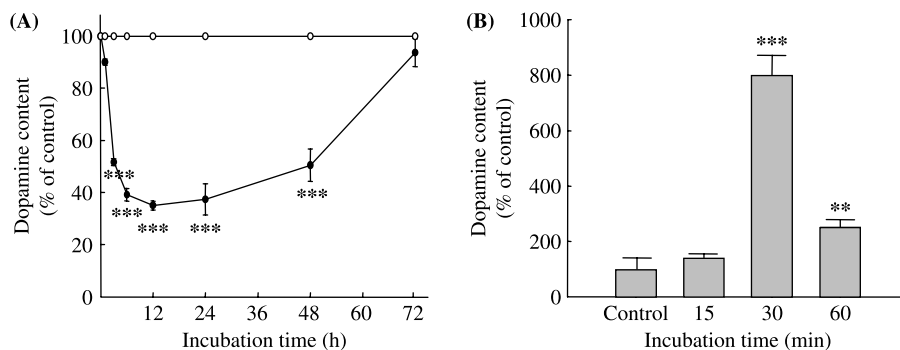


Figure 2. Time courses of the (A) intracellular and (B) extracellular dopamine content by 0.15 μ M asimilobine in PC12 cells. The control values of the intracellular and extracellular dopamine content were 3.36 ± 0.23 nmol/mg protein and 0.15 ± 0.06 nmol/min per mg protein, respectively. Results represent means \pm SEM of four experiments. Significantly different from control values: * $p < 0.05$, ** $p < 0.01$ (ANOVA followed by Tukey's test).

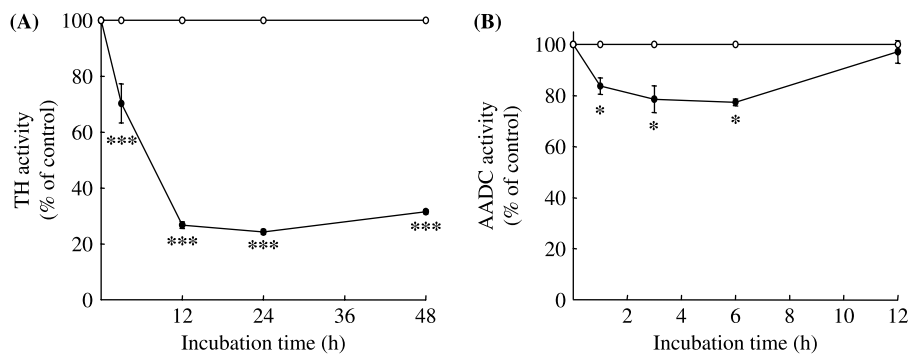


Figure 3. Time courses of (A) TH and (B) AADC activities by asimilobine (0.15 μ M) in PC12 cells. The control values of the activities of TH and AADC were 3.88 ± 0.24 and 3.65 ± 0.22 nmol/min per mg protein, respectively. Results represent means \pm SEM of four experiments. Significantly different from control values: * $p < 0.05$, ** $p < 0.01$ (ANOVA followed by Tukey's test).

PC12 rat adrenal pheochromocytoma cell lines have dopamine synthesizing, storing, and releasing properties similar to those of the neurons [28]. The PC12 cells also express the catecholamine bio-

synthetic enzymes such as TH, AADC, and dopamine β -hydroxylase (EC 4.14.17.1) [28].

In this study, therefore, the effects of asimilobine on dopamine biosynthesis and

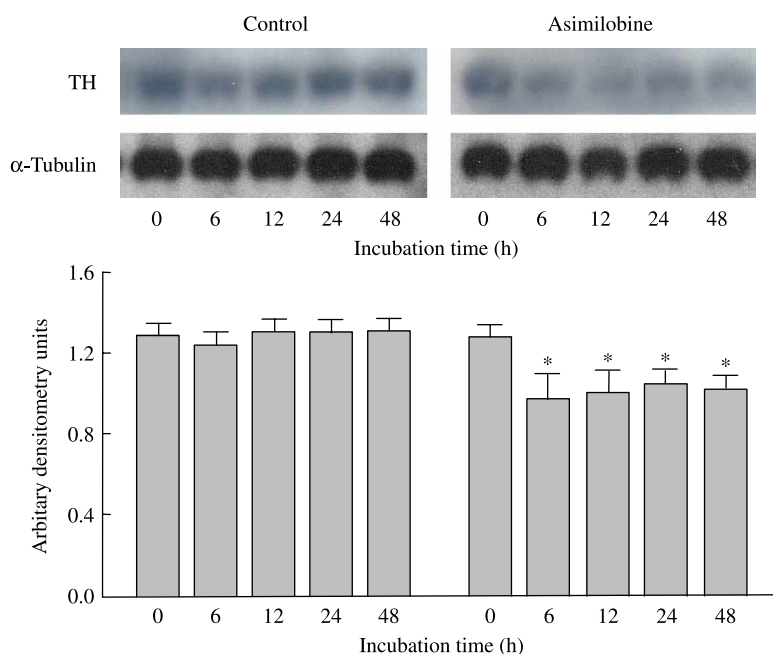


Figure 4. Effects of asimilobine on TH mRNA levels in PC12 cells. RNA was extracted and 10 μ g aliquots were subjected to electrophoresis on formaldehyde gels, blotted onto a nylon membrane and probed with 32 P-labeled cDNA probes for rat TH and α -tubulin. Equal loading of the gels was verified by ethidium bromide staining of total RNA. Relative densitometry ratio in control was expressed as 1 arbitrary unit (means \pm SEM, $n = 3$). Significantly different from control values: * $p < 0.05$ (ANOVA followed by Tukey's test).

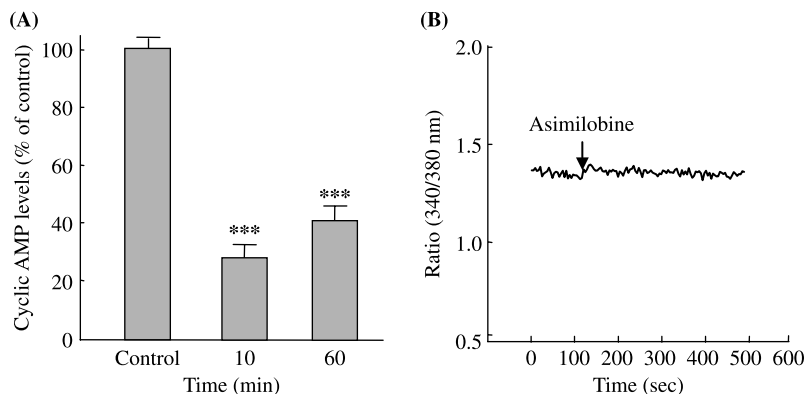


Figure 5. Effects of asimilobine on cyclic (A) cyclic AMP levels and (B) Ca^{2+} concentrations in PC12 cells. The PC12 cells were treated with asimilobine ($0.15 \mu\text{M}$) and incubated at 37°C for 10 min. Intracellular cyclic AMP levels were measured by an enzyme immunoassay kit. The PC12 cells were also loaded with Fura-2 AM for 10–60 min, and the basal Ca^{2+} concentration was assayed as described in Materials and methods. Cyclic AMP levels of control were $248.6 \pm 14.5 \text{ pmol/mg}$ protein. Results represent means \pm SEM of five to seven wells. Significantly different from control values: *** $p < 0.001$ (ANOVA followed by Tukey's test).

L-DOPA-induced cytotoxicity in PC12 cells as a model system were investigated.

increased to 801% of control by $0.15 \mu\text{M}$ asimilobine at 30 min (Figure 2(B)).

2. Results and discussion

2.1 Time courses of intracellular and extracellular dopamine levels

The intracellular and extracellular dopamine levels in normal PC12 cells were $3.36 \pm 0.23 \text{ nmol/mg}$ protein and $0.15 \pm 0.06 \text{ nmol/min}$ per mg protein, respectively. In these conditions, the activities of TH and AADC were also 3.88 ± 0.24 and $3.65 \pm 0.22 \text{ nmol/min}$ per mg protein, respectively.

Asimilobine at concentration ranges of 0.05 – $0.2 \mu\text{M}$ reduced the intracellular dopamine levels of PC12 cells after 24 h exposure (58.2% inhibition at $0.15 \mu\text{M}$). The IC_{50} value of asimilobine was $0.13 \mu\text{M}$. The dopamine content decreased at 1 h after exposure to $0.15 \mu\text{M}$ asimilobine and reached minimal levels about 35.1% of control levels at 12 h (Figure 2(A)). Subsequently, the decreased dopamine levels started to recover at 24 h and reached the same control levels at 72 h. The extracellular dopamine levels, which were secreted from the intracellular dopamine, were

2.2 Time courses of TH and AADC activities

Asimilobine at $0.15 \mu\text{M}$ inhibited TH activity to 27.8% of control levels at 12–48 h (Figure 3(A)), and then the TH activity recovered to 64.5% of control levels at 72 h (data not shown). The AADC activity was also reduced by $0.15 \mu\text{M}$ asimilobine to 87.5% of control levels at 3 h and then recovered rapidly at about 12 h (Figure 3(B)). The TH activity was more markedly inhibited by asimilobine for a longer period than the AADC activity.

2.3 TH mRNA levels

TH mRNA levels were decreased to approximately 65–68% of control levels at about 3–24 h after exposure to $0.15 \mu\text{M}$ asimilobine (Figure 4), and then slowly recovered to approximately 72% of control levels at 48 h when treated with $0.15 \mu\text{M}$ asimilobine. The inhibitory patterns were similar to those of dopamine levels.

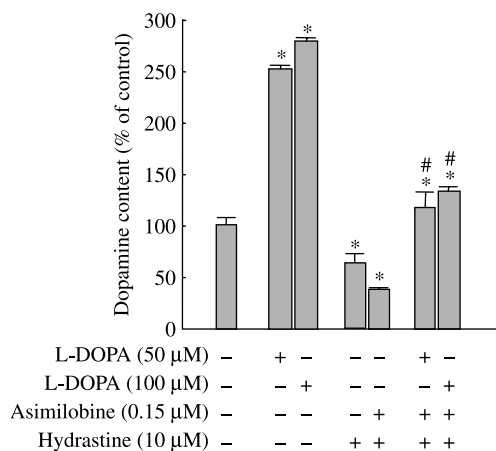


Figure 6. Effects of asimilobine on the L-DOPA-induced dopamine content in PC12 cells. The PC12 cells were exposed to L-DOPA (50 and 100 μM) in the absence or presence of asimilobine (0.15 μM) for 24 h. The control value of the dopamine content was 3.57 ± 0.24 nmol/mg protein. (1*R*,9*S*)- β -Hydrastine at 10 μM was used for the positive control.¹⁷ Results represent means \pm SEM of four experiments. * $p < 0.01$ compared with control values; # $p < 0.05$ compared with the corresponding L-DOPA concentrations (ANOVA followed by Tukey's test).

2.4 Cyclic AMP levels and Ca^{2+} concentrations

Intracellular cyclic AMP levels were significantly reduced by asimilobine at 0.15 μM to

26.4% of control levels at 10 min, and then recovered to 39.5% of control levels at 1 h (Figure 5(A)). However, asimilobine at 0.15 μM did not alter the basal Ca^{2+} concentrations in Ca^{2+} -free solution (Figure 5(B)).

2.5 L-DOPA-induced dopamine levels

Treatment with L-DOPA (50 and 100 μM) alone increased the intracellular dopamine levels up to 252.8 and 279.5% for 24 h when compared with control levels, respectively (Figure 6) [12]. Asimilobine at a non-cytotoxic concentration of 0.15 μM significantly decreased the intracellular dopamine levels to 135.1–147.4% when compared with L-DOPA alone (Figure 6). Hydrastine (10 μM) was used as a positive control [17]. Similar patterns were also observed at 48 h exposure to 0.15 μM asimilobine (data not shown).

2.6 L-DOPA-induced cytotoxicity

Asimilobine at concentrations up to 5.0 μM was not cytotoxic toward PC12 cells. However, at concentrations higher than 10 μM , asimilobine caused cytotoxicity (data not shown). L-DOPA at 100 μM for 24 h and at 50 μM for 48 h caused a reduction

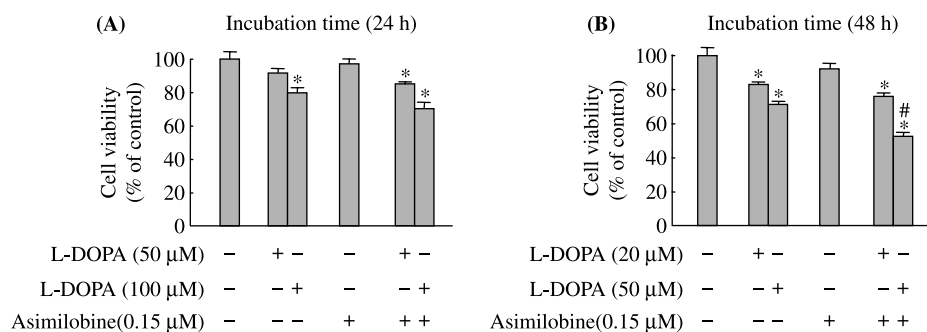


Figure 7. Effects of asimilobine on L-DOPA-induced cell viability in PC12 cells. The PC12 cells were exposed to L-DOPA (20, 50, and 100 μM) in the absence or presence of asimilobine (0.15 μM) for (A) 24 and (B) 48 h. The cell viability was assessed by the conversion of the MTT assay to insoluble blue formazan crystals. Results represent means \pm SEM of four experiments. * $p < 0.05$ compared with control values; # $p < 0.05$ compared with the corresponding L-DOPA concentrations (ANOVA followed by Tukey's test).

in the cell viability to 79.5 and 71.2% of control levels, respectively (Figure 7(A),(B)) [12]. Asimilobine (0.15 μM) associated with L-DOPA (50 and 100 μM) for 24 h significantly decreased cell viability to 91.5–81.2% when compared with L-DOPA alone (Figure 7(A)). Furthermore, when asimilobine (0.15 μM) was associated with L-DOPA (20 and 50 μM) for 48 h, the cell viability showed a more significant reduction compared with L-DOPA alone (Figure 7(B)).

2.7 Discussion

Isoquinoline derivatives have been proved to regulate dopamine biosynthesis and catabolism [13–20]. Among them, aporphine isoquinoline alkaloids such as bulbocapnine, liriodenine, and anonaine show the inhibitory effects on dopamine biosynthesis by reducing the TH activity in PC12 cells [14,21]. Asimilobine, liriodenine, and anonaine have a similar molecular structure at the aporphine ring (Figure 1).

In this study, 0.05–0.2 μM asimilobine significantly inhibited dopamine biosynthesis and L-DOPA-induced increases in dopamine levels in PC12 cells. The TH and AADC activities were also inhibited by 0.15 μM asimilobine; the inhibition of TH activity was stronger and longer than that of AADC activity (Figure 3). Asimilobine also induced the extracellular dopamine release at early time points (30–60 min; Figure 2(B)). Therefore, asimilobine reduced the intracellular dopamine levels by inhibiting dopamine biosynthesis and by inducing dopamine release in PC12 cells.

TH activity and TH gene transcription are regulated by the intracellular cyclic AMP and Ca^{2+} levels [5]. In this study, 0.15 μM asimilobine reduced TH mRNA levels (Figure 4) and also reduced the intracellular cyclic AMP levels, but not the basal Ca^{2+} concentrations (Figure 5). These results indicate that asimilobine inhibits dopamine biosynthesis by mainly mediating TH activity and TH gene expression through cyclic AMP in PC12 cells.

The order of inhibitory potency on dopamine biosynthesis was anonaine > asimilobine > liriodenine > bulbocapnine in comparison with their IC_{50} values (anonaine, 0.05 μM ; asimilobine, 0.13 μM ; liriodenine, 8.4 μM ; bulbocapnine 26.7 μM) [14,21]. Therefore, it is suggested that the isoquinoline ring planarity (liriodenine and anonaine/asimilobine; Figure 1) and the 11-hydroxy and methoxy groups of the aporphine alkaloid structure (bulbocapnine and liriodenine/anonaine/asimilobine) play a key role in the inhibition of dopamine biosynthesis in PC12 cells.

It has been found that the cytotoxic action of L-DOPA is mediated by ROS associated with oxygen free radicals and quinone derivatives [10,11]. L-DOPA at 20–100 μM increases the intracellular dopamine levels after 24–48 h in PC12 cells [12,17]. However, at concentrations higher than 50 μM for 48 h, L-DOPA induces intracellular cytotoxicity by the oxidative stress-induced ROS formation [12,20]. It is also reported that non-hydroxylated regions in aporphine and protoberberine derivatives and phenolic hydroxy groups in benzyloisoquinolines are necessary for the inhibition of peroxidation [23]. Phenolic aporphines are thought to scavenge ROS by generating stable phenoxy radicals, and nonphenolic analogues also act through similarly stabilized benzylic free radicals centered at C-6a [25]. The phenolic aporphine alkaloid such as bulbocapnine has antiperoxidative [23] and dopamine D_1 receptor antagonistic effects [22]. Boldine also exhibits antioxidant effects and attenuates dopamine- and 6-hydroxydopamine-induced oxidative damage to brain mitochondria and PC12 cells [24,29]. In addition, glaucine, the nonphenolic dimethyl ether of boldine, shows a potent antioxidant activity [27].

In the present study, when asimilobine (0.15 μM) was associated with L-DOPA (50–100 μM), the cell viability was significantly enhanced when compared with L-DOPA alone (Figure 7), suggesting that a phenolic aporphine alkaloid asimilobine did not show an antioxidant activity. Anonaine also

enhanced L-DOPA-induced cytotoxicity (submitted/data not shown). However, lirioidenine showed a mild protective activity against L-DOPA (50–100 μ M)-induced cell death at 24–48 h in PC12 cells [21]. Lirioidenine significantly decreased the basal Ca^{2+} concentration in Ca^{2+} -free solution [21]; however, asimilobine and anonaine did not alter it (Figure 5(B)). The differences of the cytotoxicological functions in aporphine derivatives need to be studied further.

In conclusion, asimilobine inhibited dopamine biosynthesis and L-DOPA-induced increases in dopamine levels by mainly reducing the TH activity and TH mRNA levels in PC12 cells. Asimilobine also enhanced L-DOPA-induced cell death in PC12 cells. The present study suggests that the L-DOPA long-term therapy with ROS-inducible agents including the natural products especially can deteriorate the physiological functions of L-DOPA. The *in vivo* neurobiological toxicity by natural products remains to be fully elucidated.

3. Materials and methods

3.1 Chemicals

Asimilobine was supplied from Korea Research Institute of Chemical Technology [3]. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), L-tyrosine, DL-6-methyl-5,6,7,8-tetrahydropterine, L-DOPA, and Fura-2 AM were purchased from Sigma Chemical Co. (St Louis, MO, USA). Cyclic AMP enzyme immunoassay system kit was purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). All sera, antibiotics, and RPMI-1640 medium for cell culture were obtained from Gibco (Grand Island, NY, USA). All the other chemicals were of reagent grade.

3.2 Cell culture

The 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 μ l/ml streptomycin. The cells were maintained in a humidified atmosphere with 5% CO_2 and 95% air at 37°C [28].

3.3 Determination of dopamine levels

Intracellular and extracellular dopamine levels were analyzed by an HPLC system [13,17]. Trichloroacetic acid (1 M, 100 μ l) and isoproterenol (100 pmol, internal standard) were added to the pellet extracts. The mixture was passed through a Toyopak SP-M cartridge (Na^+ , resin 1 ml, Toso, Tokyo, Japan) and the cartridge eluate was derivatized with 1,2-diphenylethylenediamine. The final reaction mixture was injected into an HPLC system (Toso). The analysis conditions of HPLC were the same as previously described [13]. The protein content was determined using a bovine serum albumin as a standard [30].

3.4 Assay for TH and AADC activities

TH activity was measured by a slightly modified procedure as previously described [13,19]. The enzyme reaction was performed at 37°C for 8 min in the presence of tyrosine and DL-6-methyl-5,6,7,8-tetrahydropterine. The reaction mixture was passed through an alumina cartridge and the cartridge eluate was injected into an HPLC equipped with a CM8010 electrochemical detector (Toso). The HPLC analysis was performed as previously described [19]. AADC activity was measured according to the method of Shin *et al.* [13,17] Dopamine, formed from the enzyme reaction from a substrate L-DOPA, was determined using an HPLC method.

3.5 RNA extraction and Northern blotting

Total RNA from PC12 cells was isolated, and RNA samples were separated by electrophoresis and were transferred to a nylon membrane (ICN, East Hills, NY, USA). The Northern blot analysis for TH mRNA was

performed according to the method of Kim *et al.* [5] The blots were hybridized to the coding regions of the 0.7 kb rat TH cDNA probe labeled with [α - 32 P] dCTP using a random primer labeling system (Dupont NEN, Boston, MA, USA).

3.6 Measurement of cyclic AMP levels

Intracellular cyclic AMP levels were measured by using a cyclic AMP enzyme immunoassay system kit. Briefly, the PC12 cells were incubated for a suitable time and agitated after lysis reagent was added. Finally, the absorbance was determined using a Bauty Diagnostic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm within 30 min.

3.7 Measurement of Ca^{2+} concentrations

The PC12 cells were washed with modified Krebs–Ringer–HEPES solution, and then loaded with 5 μ M Fura-2 AM at 37°C for 30–60 min. Loaded cells were resuspended and transferred into a quartz cuvette. The fluorescence intensity was measured with double excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm (Luminescence Spectrometer, Ratio Master PTI, Brunswick, NJ, USA).

3.8 Measurement of cell viability: MTT assay

The cell viability was assayed by the conventional MTT method [12,20]. The MTT solution was added to each well and incubated for 4 h at 37°C. The reaction mixtures were added to the acidic isopropanol to dissolve through mixing. The absorbance was measured by using a Bauty Diagnostic Microplate Reader (Molecular Devices) at 570 nm.

3.9 Statistical analysis

All data were presented as means \pm SEM of at least four experiments. Statistical analysis

was performed using ANOVA followed by Tukey's test.

Acknowledgements

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