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Inhibition of Tyrosine Hydroxylase by (1R,9S)-β-Hydrastine Hydrochloride in PC12 cells

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Abstract – It is reported that (1R,9S)-β-hydrastine hydrochloride (BHSH) decreased the intracellular dopamine content by inhibiting tyrosine hydroxylase (TH) activity in PC12 cells. In this study, the inhibitory mechanisms on TH activity by BHSH in PC12 cells were investigated. BHSH treatment caused a reduction of TH activity and TH mRNA level in a dose-dependent manner. After the treatment of 20 μM BHSH, TH activity and TH mRNA content were reduced at 15 min, reached the minimal levels at 6-24 h, and then recovered gradually to the control level. BHSH at 10-50 μM caused a decrease in the basal intracellular cyclic AMP levels at 10 min in a concentration-dependent manner. In addition, BHSH at 20-100 μM decreased the basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) immediately in a dose-dependent manner. BHSH also inhibited the 56 mM K^+ depolarization-induced elevation in $[Ca^{2+}]_i$, and blocked caffeine-activated store-operated Ca^{2+} entry in PC12 cells. These data suggest that BHSH inhibits TH activity and TH gene expression, in part, through reducing cyclic AMP content and basal $[Ca^{2+}]_i$ in PC12 cells.
Keywords – (1R,9S)-β-Hydrastine hydrochloride; Tyrosine hydroxylase; Cyclic AMP; $[Ca^{2+}]_i$; PC12 cells

Introduction

(1R,9S)-β-Hydrastine is a phthalide isoquinoline alkaloid and has been isolated from the roots of *Hydrastis canadensis* L. (Ranunculaceae), *Berberis laurina* Billb. (Berberidaceae) and *Corydalis stricta* Steph. (Papaveraceae) (Fang *et al.*, 1981). Recently, it is proved that (1R,9S)-β-hydrastine hydrochloride (BHSH) decreases the intracellular dopamine content by inhibiting tyrosine hydroxylase (TH, E 1.14.16.2), the first and major rate limiting enzyme in catecholamine biosynthesis, in PC12 cells (Kim *et al.*, 2001), and also inhibits bovine adrenal TH activity (Lee *et al.*, 1997). However, the mechanisms of BHSH on down-regulated TH are unclear.

PC12 rat adrenal pheochromocytoma cells have dopamine synthesizing, storing and releasing properties similar to those of the neurons (Byrd *et al.*, 1986; Gebreyesus, 1993). PC12 cells also express the catecholamine biosynthetic enzymes such as TH, aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) and dopamine β-hydroxylase (EC 1.14.17.1) (Tischler *et al.*, 1983; Byrd *et al.*, 1986; Gebreyesus, 1993), and possess many important neurochemical and signal transduction processes in a manner similar to dopaminergic neurons (Shafer and Atchison, 1991).

In the present study, the regulatory mechanisms of BHSH on TH activity were investigated using PC12 cells.

Materials and Methods

Materials – (1R,9S)-β-Hydrastine hydrochloride, catalase, L-tyrosine, dopamine, HEPES, DL-6-methyl-5,6,7,8-tetrahydropterine and fura-2 AM were purchased from the Sigma Chemical Company (St. Louis, Mo, USA). Cyclic AMP enzyme immunoassay kit was supplied from the Amersham Pharmacia Biotech (St. Freiburg, Germany). All sera, antibiotics and RPMI 1640 for cell cultures were obtained from Gibco (Grand Island, NY, USA). All other chemicals were of reagent grade.

Cell culture – PC12 cells were routinely grown in RPMI medium 1640 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 (Tischler *et al.*, 1983). After being

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Abbreviation used: TH: Tyrosine hydroxylase; BHSH: (1R,9S)-β-Hydrastine hydrochloride; $[Ca^{2+}]_i$: Intracellular Ca^{2+} concentration; SOC: Store-operated Ca^{2+}.

treated with BHSH and incubated for indicated time, PC12 cells were harvested and the cell pellets were used for the experiments.

Determination of dopamine content – Dopamine content was determined as described previously (Mitsui *et al.*, 1985; Shin *et al.*, 2000). Trichloroacetic acid (1 M, 100 μl) and isoproterenol (1 nmol/ml, 100 μl, internal standard) were added to the pellet extract. 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 (100 μl) was injected into an HPLC system (Toso). The HPLC conditions were the same as described previously (Shin *et al.*, 2000).

Assay for TH activity – TH activity was measured according to the slightly modified procedure of Nagatsu *et al.* (1979) as described previously (Shin *et al.*, 2000). L-DOPA, which was produced by enzyme reaction of 10 min from a substrate L-tyrosine, was determined using an HPLC equipped with a CM8010 electrochemical detector (Toso). The HPLC analysis was performed as described previously (Shin *et al.*, 2000).

Determination of TH mRNA content – The total RNA was extracted from PC12 cells using the modified phenol-guanidium isothiocyanate method as described by Chomczynski and Sacchi (1987). The Northern blot analysis for TH mRNA was performed according to the method of Kim *et al.* (1993b). 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).

Measurement of cyclic AMP level – PC12 cells (1×10^4-10^6 cells/ml, 100 μl in 96-well plate) were incubated for 10 min at 37°C after replaced culture medium with novel medium containing BHSH. The reaction was terminated by replacing media with lysis buffer followed by incubation on ice for 10 min. Cyclic AMP levels in the cells were measured using an enzymeimmunoassay system kit (Amersham Pharmacia Biotech, St. Freiburg, Germany).

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$) – PC12 cells (3×10^6 cells/ml) were loaded with 4 μM Fura-2 AM at 37°C for 30-60 min to determine $[Ca^{2+}]_i$ as previously described (Kim *et al.*, 2001). The cells were transferred into a quartz cuvette and the fluorescence intensity was measured with a dual excitation of 340 nm and 380 nm, and an emission of 510 nm (Ratio Master PTI, Brunswick, NJ, USA).

Statistical analysis – Protein amounts were determined with the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. All data were expressed as means± S.E.M. of at least four or five experiments. Statistical analysis was performed using ANOVA followed by Tukey's test.

Results and Discussion

The previous study indicated that BHSH treatment caused a decrease in the basal intracellular dopamine content by the inhibition of intracellular TH activity, without altering the extracellular dopamine content and inducing the cell death in PC12 cells (Kim *et al.*, 2001). In this study, therefore, the time courses for dopamine content, TH activity and TH mRNA levels by BHSH treatment were investigated.

As shown in Fig. 1, dopamine content continuously decreased for the first 3 h with the treatment of 20 μM BHSH, and reached the minimal level of about 37% of control at 18 h. The decreased dopamine level remained unaltered up to 36 h, and then gradually returned to the control level by 72 h. In addition, BHSH treatment did not affect the cell viability and cell numbers towards PC12 cells.

The temporal pattern of TH activity by BHSH in PC12 cells were determined. TH activity was rapidly decreased at 15 min to the minimal level of about 55% of control after the treatment with 20 μM BHSH, and remained low up to 12-24 h, and then recovered gradually to the control level at 36-48 h (Fig. 2A). In these conditions, TH mRNA levels decreased at about 6 h after 20 μM BHSH treatment, reached a minimal level at 24 h, and then returned toward the control level at 36-48 h (Fig. 2B).

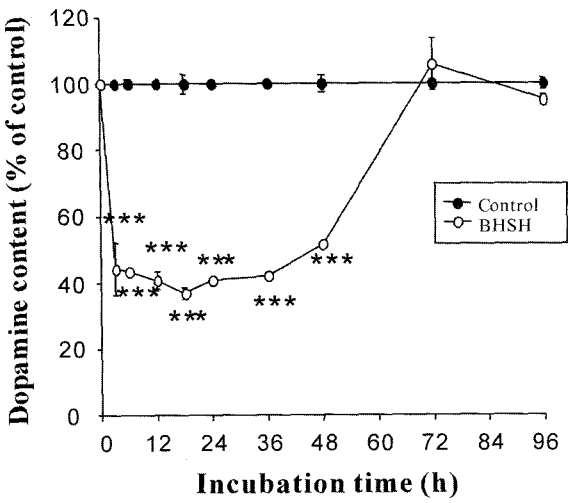

Fig. 1. Time course of dopamine content by BHSH (20 μM) in PC12 cells. The control value of dopamine content was 3.82±0.27 nmol/mg protein. Results represent the means±S.E.M. of five experiments performed in triplicate. ***, $P<0.001$ compared with the control (ANOVA followed by Tukey's test).

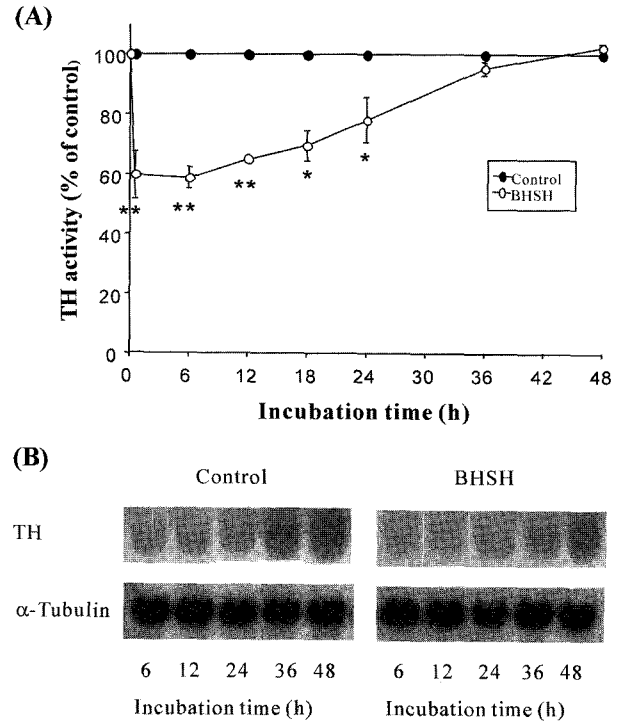

Fig. 2. Time course of TH activity by BHSH (20 μM) in PC12 cells (A). The control value of TH activity was 3.81±0.18 nmol/min/mg protein. Results represent the means±S.E.M. of five experiments performed in triplicate. *, P<0.05; **, P<0.01 compared with the control (ANOVA followed by Tukeys test). Time course of TH mRNA level by BHSH in PC12 cells (B). RNA was extracted and 10 μg aliquots were subjected to electrophoresis on formaldehyde gels, blotted onto nylon, and prebed 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.

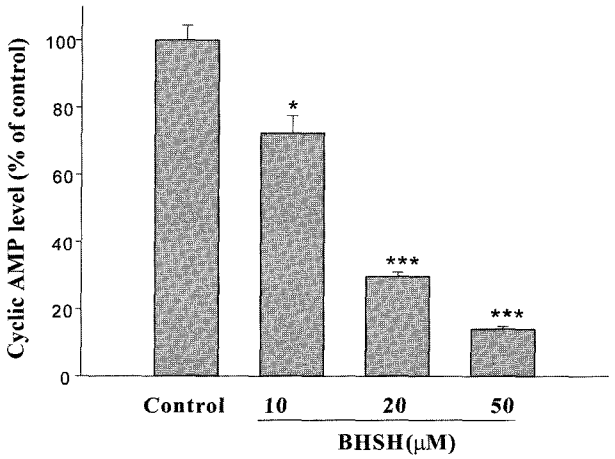

Fig. 3. Effects of BHSH on cyclic AMP levels in PC12 cells. The cells were treated with BHSH (10, 20, and 50 μM) for 10 min and the intracellular cyclic AMP content was measured by EIA. The cyclic AMP level of the control was 3.84±0.32nmol/mg protein. Results represent the means ± S.E.M. of five experiments performed in triplicate. *, P<0.05; ***, P<0.001 compared with the control (ANOVA followed by Tukey's test).

Numerous previous studies provide compelling evidence that intracellular cyclic AMP- and Ca^{2+}-mediated pathways might be important for regulating TH activity and mRNA level. Cyclic AMP enhances TH activity and TH transcription (Fossom *et al.*, 1992; Kim *et al.*, 1993b), in part, via binding of transcription factors to cyclic AMP-response elements (CRE) (Kim *et al.*, 1993b; Best *et al.*, 1995). $[Ca^{2+}]_i$ can also alter the TH activity and gene transcription in catecholaminergic cells by Ca^{2+}-related secondary signal pathways such as protein kinase A (PKA) (Caldwell *et al.*, 1992), Ca^{2+}/calmoduin kinase II (Braun and Schulman, 1995; MacNicol and Schulman, 1992) and Ca^{2+}- and phospholipid-dependent protein kinase (PKC) (TerBush and Holz, 1986).

BHSH at 10-50 μM significantly reduced the level of cyclic AMP in a concentration-dependent manner (Fig. 3). The 20 μM BHSH treatment decreased cyclic AMP content by about 61% compared to control cultures.

BHSH at concentration ranges of 20-100 μM also caused a significant decrease in the basal $[Ca^{2+}]_i$ in a concentration-dependent manner (data not shown). In the presence of external Ca^{2+}, 56 mM K^+ treatment causes a rapid elevation of $[Ca^{2+}]_i$ due to membrane depolarization-induced Ca^{2+} influx through voltage-gated Ca^{2+} channel, and caffeine stimulation also causes a biphasic rise in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from caffeine/Ry-sensitive stores and the store-operated Ca^{2+} (SOC) entry (Koizumi and Inoue, 1998). In this study, BHSH at 50 μM decreased the elevation of $[Ca^{2+}]_i$ elicited by 56 mM K^+ (Fig. 4A), and the sustained rise in $[Ca^{2+}]_i$ after elevation of $[Ca^{2+}]_i$ by caffeine (Fig. 4B),

Elevation of cyclic AMP by forskolin and activation of PKA trigger increases of TH mRNA, however, in PKA deficient cell lines, cyclic AMP analogs do not enable induction of TH mRNA (Fossom *et al.*, 1992; Mcmahon and Sabban, 1992; Kim *et al.*, 1993b). The bradykinin or thapsigargin treatment elicits Ca^{2+} release from Ca^{2+} store, results in elevation of TH mRNA levels, however, BAPTA, a high affinity chelator of $[Ca^{2+}]_i$, prevents the elevation of $[Ca^{2+}]_i$ and TH mRNA levels elicited by bradykinin or thapsigargin (Rodland *et al.*, 1990; Menezes *et al.*, 1996). Exposure of PC12 cells to 50 mM K^+ or veratridine in isotonic conditions elicits an elevation in steady state TH mRNA levels (Kilbourne and sabban, 1990).

The previous experiment indicated that BHSH does not alter intracellular cyclic AMP and TH mRNA levels in PC12 cells (Kim *et al.*, 2001). The discrepancy was attributed to the difference in determination time. In the previous experiments, the levels of cyclic AMP and TH mRNA were determined only at 24 h, while as seen in these experiments cyclic AMP level started to decrease at 10 min, and the decreased TH mRNA by BHSH initiated to recover at 24 h

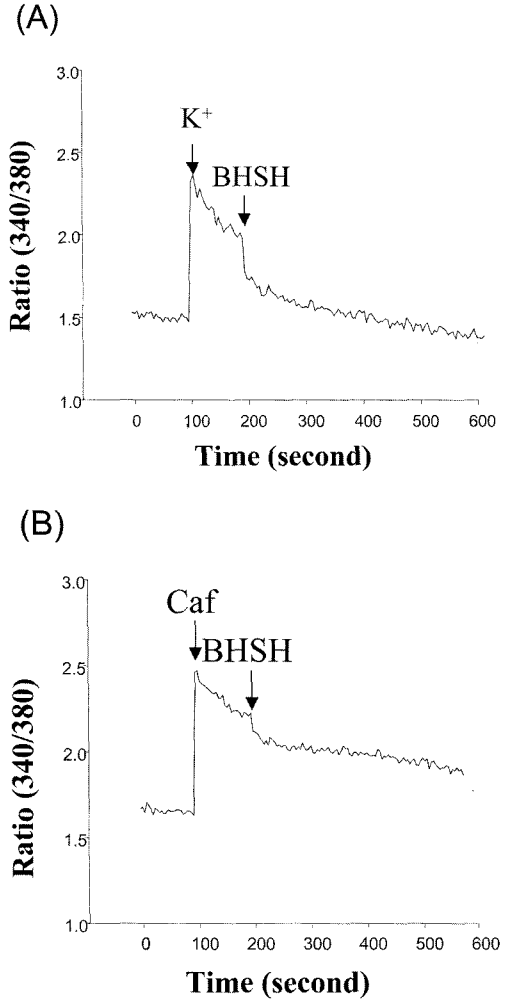

Fig. 4. Effect of BHSH on $[Ca^{2+}]_i$ in the presence of external Ca^{2+} in PC12 cells. As indicated by the arrow, 56 mM K^+ alone or 50 μM BHSH after elevation of $[Ca^{2+}]_i$ elicited by 56 mM K^+ (A), 20 mM caffeine (Caf) alone or 50 μM BHSH after elevation of $[Ca^{2+}]_i$ elicited by 20 mM caffeine (B) were added to the cells. The cells had been loaded with Fura-2 AM for 30-60 min, and $[Ca^{2+}]_i$ were assayed as described in Materials and Methods. The data shown are representative tracings from three independent experiments.

(Fig. 2B).

Taken together, the present study showed that BHSH treatment caused a decrease in the basal intracellular cyclic AMP levels in PC12 cells. BHSH also decreased the basal $[Ca^{2+}]_i$ immediately, and blocked the high K^+-induced voltage-gated Ca^{2+} channel and the caffeine-activated SOC entry in PC12 cells. The reductions of the intracellular cyclic AMP level and basal $[Ca^{2+}]_i$ by BHSH are contributed to the down-regulation of TH, in part, resulting in the inhibition of dopamine biosynthesis in PC12 cells. The pharmacological functions of BHSH need to be studied further.

Acknowledgements

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