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NERVE GROWTH FACTOR TRANSIENTLY INCREASES TETRAHYDROBIOPTERIN AND TOTAL BIOPTERIN CONTENTS OF PHEOCHROMOCYTOMA PC12h CELLS*

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SUMMARY: Nerve growth factor (NGF) is known to induce differentiation of pheochromocytoma into sympathetic neuron-like cells. Tetrahydrobiopterin (BPH_4) and total biopterin (BP) levels in PC12h, a subclonal line of PC12, were transiently increased by NGF: the increase in BPH_4 and BP reached the maximum (20-25 ng/mg protein=about 2-fold over the control level) at 24 h after the treatment was started. After 2-3 days, the BPH_4 and BP levels decreased to the same level as in control cells. The NGF concentration which gave a half maximal BP increase by 24 h-treatment was around 1 ng/ml.

Tetrahydrobiopterin (BPH_4) is known to serve as a specific cofactor (electron donor) for aromatic amino acid hydroxylases (see review see Ref.1), such as tyrosine hydroxylase [EC 1.14.16.2] which is thought to be the rate-limiting enzyme in the biosynthetic pathway for catecholamine neurotransmitters. Nerve growth factor (NGF) is a protein required for the survival and maintenance of sympathetic and sensory neurons (2). Furthermore, NGF has been thought to also have vital role in cells involved in central nervous system (3). In in vitro culture system, PC12 pheochromocytoma cells which resemble their nontumor counterparts adrenal chromaffin cells, are induced to differentiate into sympathetic neuron-like cells by NGF (4) and, therefore, widely used for studying the action mechanisms of NGF (5,6). PC12 cells are also used for the studies on tyrosine hydroxylase and on catecholamine metabolism (5). However, the effect of NGF on tetrahydrobiopterin (BPH_4), an essential cofactor for tyrosine hydroxylase, has not been clarified yet.

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Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor; dBcAMP, N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate; BP, biopterin; BPH_4, 5,6,7,8-tetrahydrobiopterin; PCA, perchloric acid; HPLC, high-performance liquid chromatography.

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In the present paper, we examine the effect of NGF on total BP and BPH_4 levels in PC12h cells. PC12h is a subclonal line of PC12 pheochromocytoma (7) and has almost the same properties as PC12 except that in PC12h tyrosine hydroxylase is induced by NGF (7,8). The treatment of PC12h cells with NGF results in the transient increase in both BPH_4 and total BP contents in the cells. This effect is not observed with NGF concentrations lower than 0.1 ng/ml, and reaches to the plateau level at around 5 ng/ml. Dibutyryl cyclic AMP (dBcAMP) also shows the similar effect as NGF.

EXPERIMENTAL PROCEDURES

PC12h cells were a gift from Dr. Hiroshi Hatanaka, Mitsubishi-Kasei Institute of Life Sciences. They were maintained in plastic culture dishes in the same medium as that for PC12 cells as described previously (9): Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum, 7% horse serum heat inactivated, 100 µg/ml streptomycin, and 100 units/ml penicillin. For the experiments, 2×10^5 cells were plated on 5-cm plastic culture dishes in the same medium. One or two days after plating, the culture medium was changed and NGF (usually 50 ng/ml) was added to the culture. The medium was changed once every 3 or 4 days.

Total BP content was measured using high-performance liquid chromatography (HPLC) essentially according to Fukushima and Nixon (10), and Bräutigam et al. (11): PC12h cells from a 5-cm dish were washed with 10 mM Tris-HCl, pH 7.4, containing 3 mM $CaCl_2$/1 mM $MgCl_2$/0.25 M sucrose, and were sonicated with 0.5 ml of 0.2 M perchloric acid (PCA)/10 mM dithioerythritol for 30 sec at 0°C. The sonicate was centrifuged for 15 min at 12,000 x g. The extract (the supernatant solution) was subjected to iodine oxidation under acidic conditions: to a 100 µl aliquot of the PCA extract, 50 µl of acidic iodine solution (0.5% I_2, 1% KI in 0.2 M PCA) was added and the solution was kept at room temperature for 1 h, and then 50 µl of 1% ascorbic acid (freshly prepared) was added to reduce excess iodine. A 10 µl aliquot of this solution was applied directly onto a Finepak SIL $C_{18}S$ column (4.6 x 150 mm, Japan Spectroscopic Co.) of HPLC. The column was developed by 5% (V/V) methanol/water with a flow rate of 1 ml/min. The eluate was monitored by fluorescence with excitation and emission wavelengths of 350 nm and 450 nm, respectively.

To estimate the BPH_4, another 100 µl aliquot of the PCA extract was subjected to iodine oxidation under alkaline conditions, by which BPH_4 is decomposed to another compound, 2-amino-4-hydroxypterin, whereas 7,8-dihydro and oxidized forms of BP are not (10): 25 µl of 2.5 M NaOH, and subsequently 25 µl of alkaline iodine solution (mixture of 9 vol of 1% I_2/2% KI and 1 vol of 1 M NaOH) were added, and the solution was kept for 1 h at room temperature. After the oxidation, 50 µl of 4 M PCA was added and 10 µl of this solution was analyzed for BP by HPLC. BPH_4 content was calculated by subtracting the BP content detected after the alkaline oxidation from that detected after the acid oxidation.

Precipitate fraction obtained after the centrifugation of the sonicate of the cells was washed once with 20% trichloroacetic acid, dissolved in 1 M NaOH, and used for protein measurement. Protein was determined by the method of Lowry et al. (12).

NGF (=β-NGF) was purified to homogeneity in our laboratory from submandibular glands of adult male mice by the method of Varon et al. (13). Epidermal growth factor (EGF) was obtained from Collaborative Research, Inc. (Lot 86-1427), and dBcAMP was from Sigma.

RESULTS

We examined the effect of treatment of pheochromocytoma PC12h with NGF on the BP level in the cells. As shown in Fig. 1, NGF transiently increased the total BP level in the cells. The NGF effect was detectable at least 6 h after the treatment was initiated and reached maximal level at 24 h. The extent of maximal increase in BP content was about 2-fold over the control level. After 3 days, the BP content in the NGF-treated cells became almost the same as that in the control cells.

Effect of NGF concentration on BP level was examined (Fig. 2). As the NGF concentration was increased the BP level increased and reached plateau at 5 ng/ml. A half maximal effect was observed with NGF of at around 1 ng/ml (= 37 pM), and the effect was hardly detectable with the concentrations less than 0.1 ng/ml.

Since only BPH_4 has physiological function as an electron donor for aromatic amino acid hydroxylases among the various reduced (tetrahydro, quinonoid-dihydro, and 7,8-dihydro) and oxidized forms of biopterin (1), we then examined the BPH_4 levels in the NGF-treated cells and in the control (untreated) cells by using the iodine oxidation method under alkaline conditions according to Fukushima and Nixon (10). In all cases almost all of BP in PC12h cells was present as its tetrahydro form, and when the total BP in the cells was increased by NGF, BPH_4 was also elevated (Table 1).

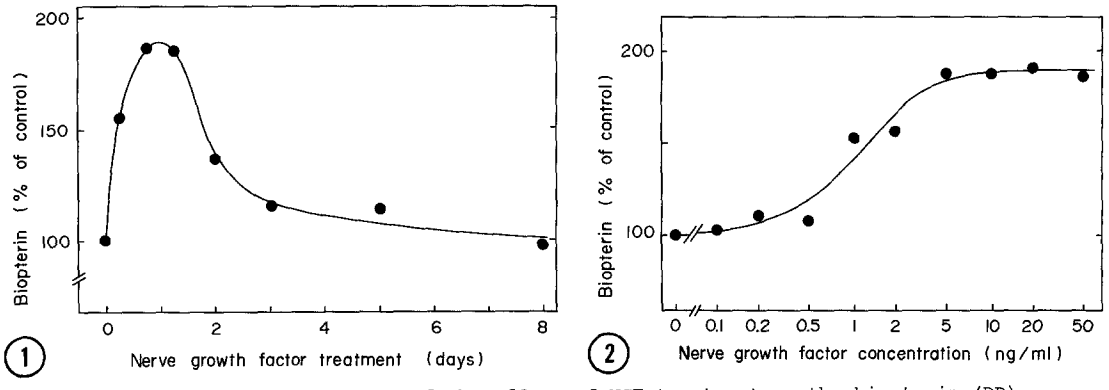

Fig. 1. Time course of the effect of NGF treatment on the biopterin (BP) level in pheochromocytoma PC12h cells. PC12h cells were cultured in 5-cm dishes in the presence of NGF (50 ng/ml) for various time periods as indicated in the figure. After the cultivation, cells were harvested and the BP content was measured as described in the text. Values are expressed as percent of BP content in control cells (see Table 1 and Table 2 for typical values) and are from the representative experiment. Similar results were obtained in additional three experiments.

Fig. 2. Effect of NGF concentration on the biopterin (BP) level of PC12h cells. PC12h cells cultured in 5-cm dishes were treated with various concentrations of NGF for 24 h, and BP content in the cells was measured as described in the text. Values are expressed as percent of BP content in control cells.

Table 1. Effect of NGF on tetrahydrobiopterin (BPH_4) and total biopterin (BP) levels in PC12h pheochromocytoma

Treatment	BP content after iodine oxidation		[a]BPH_4 content
	[b]Under acidic conditions (ng/mg protein)	[c]Under alkaline conditions (ng/mg protein)	(ng/mg protein)
Control	11.8	0.1	11.7
NGF (24 hours)	21.5	0.1	21.4
NGF (72 hours)	13.9	not detected	13.9

PC12h cells were cultured in the absence or presence of NGF (50 ng/ml). By the acidic iodine oxidation method, total BP content was determined. By the iodine oxidation under alkaline conditions, BPH_4 is decomposed to 2-amino-4-hydroxypterin, whereas 7,8-dihydrobiopterin was oxidized to BP (10). Therefore, [a]BPH_4 content was calculated by subtracting the BP content detected after the alkaline oxidation from that detected after the acid oxidation. [b,c]Values were not corrected for recovery. Recoveries of authentic BPH_4 after acid-iodine oxidation and alkaline-iodine oxidation were 87% and 5.4%, respectively. Values are from representative experiment and similar results were obtained in three additional experiments.

Several compounds which are known to give some effects on PC12h cells were tested (Table 2). EGF, which was reported to induce some common responses as NGF does (5,9), slightly increased BP level. Whereas, dBcAMP increased the BPH_4 and BP levels as in the comparable extent as NGF did.

DISCUSSION

NGF treatment of PC12h pheochromocytoma induced transient increase in BP level in the cells (Fig. 1). The NGF concentration which gave a half maximal increase in BP by 24 h treatment was about 1 ng/ml (Fig. 2), and this concen-

Table 2. Effect of various factors on the total biopterin (BP) and tetrahydrobiopterin (BPH_4) levels in PC12h pheochromocytoma cells

Treatment	Total BP content		BPH_4 content	
	(ng/mg protein)	(%)	(ng/mg protein)	(%)
None (Control)	13.1±1.65	100	12.9±1.43	100
NGF (50 ng/ml)	23.2±1.03***	177	22.1±1.04***	171
EGF (10 ng/ml)	16.2±1.10	123	15.3±0.988	119
dBcAMP (1 mM)	21.6±3.53*	165	20.3±1.18**	157

PC12h cells were cultured in the presence of various substances for 24 h, and BP and BPH_4 in the cells were measured as described in the legend for Table 1. Values are the mean±S.D. of triplicate experiments. Similar results were obtained in two additional sets of experiments. Different from respective control values at ***P<0.001; **P<0.01; and *P<0.02 (Student's t-test).

tration was well agreed with that showed a half maximal effect on neurite outgrowth from the cells (data not shown).

NGF also increased the BPH_4 level as well as BP. The majority of BP in the cells was present as its tetrahydro form and the ratio of BPH_4/BP was not changed by the NGF treatment (Table 1). With the consideration of recoveries of BP measurement, actual ratio of BPH_4/BP in the cells was estimated to be not less than 90%. Besides, biopterin was shown to be synthesized *in vivo* as a tetrahydro form (14). Therefore, the increase in BP level by NGF might be due to the increased biosynthesis of BPH_4 from GTP. Since the increase in GTP cyclohydrolase, the first enzyme in the biosynthetic pathway of BPH_4 from GTP, was usually observed when increase in BPH_4 level in certain tissues was induced by various means such as the treatment of rats with reserpine or insulin (for review see Ref.15), the cyclohydrolase seemed to be the regulatory enzyme in BPH_4 biosynthesis. That 2,4-diamino-6-hydroxypyrimidine, an inhibitor of GTP cyclohydrolase (15,16), lowered the BP level in NGF-treated cells more seriously than in control cells (N.N. and H.S., unpublished observation) suggests that, also in this case, the GTP cyclohydrolase-catalized reaction might be an important part for the induction of BP increase by NGF. In our preliminary experiments, actinomycin D also lowered the BP level in NGF-treated cells but did not affect on that in control cells.

Recently, Woolf *et al.* (17) reported that the BPH_4 level of PC12 cells was increased by treatment of the cells with dBcAMP for 24 or 48 h. In PC12h cell line, BPH_4 and BP levels were also increased by dBcAMP (Table 3). There have been contradictory observations on the NGF effect on the cAMP level of pheochromocytoma cells. Schubert and Whitlock (18) reported that NGF transiently increased the cAMP level in PC12 cells: an approximately 2-fold increase was detected at 7 min after the NGF addition, and after 22 min the cAMP level again went down to the control level. Whereas, Hatanaka *et al.* (19) did not detect the change in the cAMP level by NGF. It is yet unclear that the increase in BPH_4 and total BP by NGF is due to a mechanism in common with that by which BPH_4 is increased by the treatment of the cells with dBcAMP for 24 h, because the increase in intracellular cAMP level by NGF would not be detected (19) or, if any, be transient and decreased to the control level within 22 min of the start of treatment (18).

It is worth noting that the time course of the NGF effect on BPH_4 and total BP levels was significantly different from that of the NGF effect on tyrosine hydroxylase as described by Hatanaka (20): the increase in BPH_4 and BP was transient and the maximal effect on BP was observed by 24 h-treatment (Fig. 1), whereas tyrosine hydroxylase was continuously increased by NGF untill at least 6 days after the treatment was started (20). Thus, NGF affected BPH_4 level and tyrosine hydroxylase with quite different manner

though they are the components in the same hydroxylation system of tyrosine to dopa, suggesting some unknown physiological function(s) of BPH_4 (or BP) other than the cofactor activity for aromatic amino acid hydroxylases. Recently, Ziegler et al. (21) proposed a new function for BPH_4 as a modulator of interleukin-2 effect in peripheral blood mononuclear cells.

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