INDUCTION OF TYROSINE HYDROXYLASE SYNTHESIS IN RAT SUPERIOR CERVICAL GANGLIA *IN VITRO* BY NERVE GROWTH FACTOR AND DEXAMETHASONE

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Summary: The addition of dexamethasone and nerve growth factor to organ cultures of superior cervical ganglia from young rats induces the synthesis of tyrosine hydroxylase. The combination of nerve growth factor and dexamethasone *in vitro* produces a differential rate of tyrosine hydroxylase synthesis which approaches that obtained by the *in vivo* administration of nerve growth factor.

The most selective action of nerve growth factor (NGF) known to date is its ability to increase the specific activities of tyrosine hydroxylase (TH) and dopamine β-hydroxylase in the superior cervical ganglia (SCG) of young rats (1). It is now clear that the increased specific activity of TH is a result of an NGF-induced increase in the differential rate of synthesis of this enzyme (2). Such an increase in the rate of synthesis of TH can readily be seen in organ cultures of SCG from animals previously given NGF (2). An increase of three- to four-fold can be observed after 24 hours and a small increase can be seen as little as six hours after NGF administration (2).

In order to understand the mechanism of this action it would be desirable to have a completely *in vitro* system in which nerve growth factor induced tyrosine hydroxylase. Previous work from this laboratory has shown that, although the synthesis of TH can be measured in superior cervical ganglia *in vitro* (3), no substantial increase in the differential rate of this synthesis could be produced *in vitro* by NGF. It seems, then, that additional

Abbreviations used: NGF, nerve growth factor; TH, tyrosine hydoxylase; SCG, superior cervical ganglia; SDS, sodium dodecyl sulfate.

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factors might be necessary to obtain TH induction by NGF *in vitro*.

Work in other laboratories has implicated the glucocorticoids in the transsynaptic and the stress-related inductions of tyrosine hydroxylase in sympathetic ganglia (4-6). Recently, it has been shown that the specific activity of TH in organ cultures of SCG can be increased by the combination of nerve growth factor and dexamethasone, but that dexamethasone alone *in vitro* is ineffective (7).

This observation is of interest because it strongly suggests that the glucocorticoids are involved in the action of the nerve growth factor *in vivo*. To prove that the observed increase in the specific activity of tyrosine hydroxylase is indeed due to increased enzyme synthesis, it is necessary to show that the differential rate of TH synthesis in the ganglia is increased under these conditions. The present experiments demonstrate such an induction.

MATERIALS AND METHODS

The details of the culture conditions and the quantitative measurement of tyrosine hydroxylase synthesis have been presented recently (2,3). Briefly, superior cervical ganglia from 5-day old rats (Zivic-Miller, Allison Park, PA) were removed and decapsulated. As indicated in the Tables, some of the rats were injected subcutaneously for one or for three days with 2.5 S nerve growth factor (10 µg per gram of body weight) or intraperitoneally with dexamethasone phosphate (1.3 µmoles/kg). The ganglia were cultured in 0.35 ml of BGJ$_b$ medium, Fitton-Jackson modification, without phenol red (Grand Island Biological Company). The medium was supplemented with 0.1% bovine serum albumin, fraction V, and an antibiotic-antimycotic mixture which included penicillin, streptomycin, and fungizone at 100 units/ml, 100 µg/ml, and 0.25 µg/ml, respectively. In experiments in which TH synthesis was to be measured, 250 µCi of [^{3}H]-leucine was added to each well.

Ganglia were maintained in tissue culture clusters at 37° in a humidified atmosphere of 95% oxygen and 5% carbon dioxide for various lengths of time as specified in the Tables. At the end of the incubation the tissues were removed from culture and rinsed in 0.25 M sucrose. Each pair of ganglia was then homogenized in a ground glass homogenizer with 400 µl of 5 mM Tris, pH 7.4, containing 0.1% Triton X-100, and centrifuged at 20,000 x g for 20 minutes. The supernatant portion was used for the assay of tyrosine hydroxylase activity, the measurement of soluble protein synthesis, and the precipitation of newly synthesized, radioactive tyrosine hydroxylase.

The extent of incorporation of radioactive leucine into soluble protein was determined as follows. Five hundred µg of bovine serum albumin in 50 µl and 1 ml of 10% CCl_3COOH were added to portions of the 20,000 x g supernatant fraction. The samples were kept at 0° for 30 minutes and then filtered in a Yeda Filtering Apparatus over GF/C glass fiber paper (Whatman). The filters were washed 5 times with 5 ml of 5% CCl_3COOH and dried. The precipitates were solubilized by heating the filters at 100° for 1 hour in 1 ml of 1% SDS, and were counted in Aquasol. The efficiency of counting was 25 to 30%.

Immunoprecipitation of newly synthesized, radioactive tyrosine hydroxylase was performed in a volume of 1.0 ml in the presence of 1% Triton X-100, 1% freshly prepared sodium deoxycholate, and 200 µl of the supernatant fraction from the ganglia homogenate. Sufficient carrier TH from the 150,000 x g supernatant fluid of rat adrenal glands was added to adjust the final concentration of TH in the reaction mixtures to 2.5 units. Enough antiserum to precipitate twice the amount of TH present was then added. Samples were incubated at 30° for 1 hour and then at 4° for 16 hours. The samples were then layered onto a 0.5 ml sucrose cushion (1 M sucrose, 150 mM NaCl, 1% Triton X-100, 10 mM sodium phosphate, pH 7.0) and centrifuged at 16,000 x g for 15 minutes in an HB-4 Sorvall rotor. The top of the sucrose cushion was gently rinsed twice with phosphate-buffered saline (10 mM sodium phosphate, 140 mM sodium chloride). The pellet itself was then washed 2-3 times with phosphate-buffered saline, each time being vigorously vortexed and centrifuged for 4 minutes.

The antigen-antibody pellets were solubilized by boiling for 15 minutes in 100 µl of a solution containing 1% SDS, 1% 2-mercaptoethanol, 0.002% bromphenol blue tracking dye, and 50% glycerol. SDS-polyacrylamide gels (7.5%) were prepared according to the procedure of Weber et al. (8) and run for 5 hours at 8 mA per gel. Gels were sliced into 1.2 mm sections and the slices dissolved by heating at 55° overnight in 1 ml portions of hydrogen peroxide containing 1% ammonium hydroxide. The samples were counted in Aquasol and tyrosine hydroxylase synthesis was estimated by determining the counts in the slices representing the TH peak (2,3).

Tyrosine hydroxylase activity was determined by measuring the formation of ^3H_2O from L-tyrosine (3, 5-^{3}H) as described by Nagatsu et al. (9) and modified by Oesch et al. (10). A unit is that amount of enzyme which will produce 1 nmole of ^3H_2O per hour at 30°. Protein was determined by the method of Lowry et al. (11), using bovine serum albumin as standard.

Nerve growth factor (2.5 S form) was prepared by the method of Bocchini and Angeletti (12). Monospecific antibody against tyrosine hydroxylase was produced in sheep by repeated injections of an enzyme fragment purified from the chymotryptic digest of bovine adrenal medulla chromaffin granules (13). The γ-gluobulin fraction of the immune serum was obtained by precipitation at 40% of saturation with ammonium sulfate. The precipitated γ-globulins were reconstituted to the original volume with 20 mM potassium phosphate, pH 6.8, containing 0.3 M glycine. Prior to use the reconstituted serum was centrifuged at 12,000 x g for 4 minutes to remove insoluble protein. L-Leucine (4,5-^{3}H(N)) (specific activity:57.4 Ci/mmole) and L-tyrosine (3,5-^{3}H) (specific activity: 60.3 Ci/mmole) were obtained from the New England Nuclear Corp. Dexamethasone (Decadron) was purchased from Merck, Sharpe & Dohme. Culture media and the antibiotic-antimycotic mixture was purchased from Grand Island Biological Co. Tissue culture clusters were obtained from Microbiological Associates.

RESULTS AND DISCUSSION

The combination of nerve growth factor and dexamethasone in the culture media induces an increase in the differential rate of tyrosine hydroxylase synthesis in rat superior cervical within 24 hours (Table IA). This increase is not produced by either NGF or dexamethasone alone; in previous experiments a differential rate of between 0.10 and 0.16 has repeatedly been observed in

TABLE I

Effect of nerve growth factor and dexamethasone on the differential rate of tyrosine hydroxylase synthesis in superior cervical ganglia in vitro

	Treatment in vivo	Treatment in vitro	Soluble protein per ganglion	Radioactivity in Soluble protein	Radioactivity in TH	Synthesis of TH
			μg	cpm X 10^{-3}/mg protein		%
A.	---	NGF	28.9 ± 1.1	116,436 ± 5,344	161 ± 16	0.14 ± 0.01
	---	Dexamethasone	19.4 ± 1.1	71,425 ± 6,475	56 ± 5	0.08 ± 0.01
	---	NGF + Dexamethasone	28.0 ± 0.9	110,321 ± 12,718	305 ± 6	0.28 ± 0.03
B.	3 x NGF	NGF	55.4 ± 13.5	48,951 ± 4,848	183 ± 20	0.38 ± 0.05
	3 x NGF	NGF + Dexamethasone	50.1 ± 7.1	49,415 ± 7,772	201 ± 38	0.41 ± 0.05

Ganglia were cultured for 23-24 hours. Other conditions were as described in Materials and Methods. Each value represents the mean ± S.D. of at least three determinations. The concentration of NGF in the culture medium was 1 μg/ml; dexamethasone was present at 1 x 10^{-7}M. The experiment presented in A was with ganglia from 5-day-old animals; the ganglia in B were from 4-day-old animals which had been treated on days 1, 2, and 3 with NGF.

TABLE II

The effect of nerve growth factor and dexamethasone on the specific activity of tyrosine hydroxylase in superior cervical ganglia *in vivo*

	Treatment	Specific activity of TH units/mg protein
A.	Saline	4.77 ± 1.05 (9)
	NGF	7.60 ± 1.82 (10)
	Dexamethasone	6.07 ± 0.91 (3)
	NGF + Dexamethasone	13.89 ± 1.60 (6)
B.	3 X NGF	16.81 ± 1.62 (3)
	3 X NGF; Dexamethasone	14.16, 12.96

Each value represents the mean ± S.D. with the number of observations in parenthesis or, where individual values are given, of separate experiments. The animals in A were 5 days old when injected and 6 days old when killed. The animals in B were injected with NGF on days 1, 2, and 3 and with dexamethasone only on day 3 and were killed on day 4.

cultures to which no additions have been made (3). When the animals are treated with NGF repeatedly, a procedure which produces a maximal rate of subsequent TH synthesis *in vitro* (2), the addition of dexamethasone to the culture medium has little effect (Table IB).

The increased rate of tyrosine hydroxylase synthesis produced by the combination of nerve growth factor and dexamethasone *in vitro* is reflected in an increased specific activity of the enzyme in the ganglia. The specific activity of TH increased by 31% in 24 hours and by 52% after 48 hours in culture.

The effects of nerve growth factor and dexamethasone on tyrosine hydroxylase levels can be seen in vivo as well as in vitro. A single dose of NGF increases TH levels in ganglia, but this increase is much greater when dexamethasone is also administered (Table IIA). The effect of dexamethasone alone is minimal. When animals are injected with NGF for three consective days dexamethasone has no effect on the level of TH (Table IIB).

The mechanism of action of nerve growth factor is not known. The selective effect of NGF on the synthesis of tyrosine hydroxylase in superior cervical ganglia presents an inviting system for study. Up to now this induction of TH, easily demonstrated in vivo (1), could not be produced to the same extent in vitro. The recent work of Otten and Thoenen (7) indicates, and the present studies confirm, that corticosteroids are a part of the induction system, and addition of corticosteroids in vitro is necessary to duplicate the in vivo effect of NGF. The observation that dexamethasone has no effect when the synthesis has been induced maximally by NGF in vivo indicates that NGF and dexamethasone are acting synergistically. It seems appropriate to inspect the other actions of NGF in other systems to determine what, if any, role is played by the corticosteroids.

References

1. Thoenen, H., Angeletti, P. U., Levi-Montalcini, R., and Kettler, R. (1971) Proc. Nat. Acad. Sci., USA 68, 1598-1602.
2. MacDonnell, P., Tolson, N., and Guroff, G., in preparation.
3. MacDonnell, P., Tolson, N., Yu, M.-W., and Guroff, G., J. Neurochem., in press.
4. Otten, U. and Thoenen, H. (1975) Proc. Nat. Acad. Sci., USA 72, 1415-1419.
5. Otten, U. and Thoenen, H. (1976) Mol. Pharm. 12, 353-361.
6. Hanbauer, I., Guidotti, A., and Costa, E. (1975) Brain Res. 85, 527-531.
7. Otten, U. and Thoenen, H. (1976) Brain Res. 111, 438-441.
8. Weber, F., Pringle, J. R., and Osborn, M. (1972) Methods in Enzymology Vol. 23, (Hirs, C. H. W. and Timasheft, S. N., eds.) pp. 3-27, Academic Press, New York.
9. Nagatsu, T., Levitt, M., and Udenfriend, S. (1964) Analyt. Biochem. 9, 122-126.
10. Oesch, F., Otten, U., and Thoenen, H. (1973) J. Neurochem. 20, 1691-1706.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
12. Bocchini, V. and Angeletti, P. U. (1969) Proc. Nat. Acad. Sci., USA 64, 787- 794.
13. Lloyd, T. and Kaufman, S. (1973) Mol. Pharm. 9, 438-444.