Regulation of Nerve Growth Factor Synthesis and Release in Organ Cultures of Rat Iris

EVA-MARIA BARTH, SIGRUN KORSCHING, and HANS THOENEN
Department of Neurochemistry, Max Planck Institute for Psychiatry, D-8033 Martinsried, Federal Republic of Germany

ABSTRACT We studied the synthesis and release of nerve growth factor (NGF) in cultured rat iris with a two-site enzyme immunoassay by measuring the time course of NGF levels remaining in the iris and relased into the medium up to 72 h. For up to 3 h, the NGF levels in the iris did not change significantly. After that, they increased to a maximal level of 350 ± 30 pg NGF/iris at 19 h, which is 200 times higher than the in vivo content. Between 20 and 72 h in culture, the NGF level decreased to 130 ± 10 pg NGF/iris, whereas general protein synthesis did not change during that time period. Maximal rate of NGF production (203 pg NGF/h/iris) was seen between 9 and 12 h in culture. In the medium, NGF levels were first detectable after 6 h. Levels then increased with a time course similar to that seen within the iris, reaching a maximal level of 1,180 ± 180 pg after 19 h in vitro, and then did not significantly change for up to 48 h. The NGF production of the densely sympathetically innervated dilator was three times higher than that of the predominantly cholinergically innervated sphincter.

The NGF production was blocked by inhibitors of messenger RNA synthesis (actinomycin D) and of polyadenylation (9-β-D-arabinofuranosyladenine) as well as by inhibitors of translation (cycloheximide). Monensin, which interferes with the transport of proteins through the Golgi apparatus, decreased NGF levels to 8–12% of controls in the medium, suggesting that the Golgi apparatus is involved in the intracellular processing of NGF.

Nerve growth factor (NGF)[1] is a protein essential for the development and maintenance of function of the peripheral sympathetic and part of the sensory nervous sytems (for review, see references 1–3). NGF has been shown to be taken up with high selectivity by sympathetic and sensory nerve terminals and to be transported retrogradely to the corresponding neural perikarya (4). The interruption of the retrograde axonal transport by surgical or pharmacological procedures has the same effect as the neutralization of endogenous NGF by specific antibodies, i.e., impaired neuronal function in fully differentiated neurons and degeneration of the corresponding neurons during early stages of development (2, 5, 6).

The role of NGF as a retrograde neurotrophic messenger was based on this indirect evidence, but has recently been substantiated by the direct demonstration of retrograde axonal transport of endogenous NGF and its quantitative determination in intact sympathetically innervated target tissues (7, 8). It was established that a correlation exists between the NGF levels and the density of sympathetic innervation of target tissues (8). However the rates of NGF synthesis could not be deduced from these experiments because the NGF levels in vivo are the result of a variety of determinants: in addition to the rate of synthesis of NGF, both the efficient uptake and removal by retrograde axonal transport, diffusion into adjacent tissues, and subsequent removal by the blood stream have to be taken into account, as well as local proteolytic degradation.

We therefore decided to study the synthesis of NGF in an organ culture system, where more precise information about the various factors involved in the regulation of NGF synthesis in target tissues can be obtained. We have studied the time course of NGF synthesis and release in the cultured rat iris, a sympathetic target organ in which NGF has been shown to be present (8, 9). Moreover, we have investigated the level of regulation of the NGF synthesis and the possible intracellular processing pathways.

[1] *Abbreviations used in this paper*: ara-ade, 9-β-D-arabinofuranosyladenine; NGF, nerve growth factor.

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MATERIALS AND METHODS

Preparation of the Iris: Wistar rats (100–200 g, both sexes) were decapitated. The eye bulbs were immediately dissected out and divided 1–2 mm behind the corneo-scleral junction with razor blades, and the anterior halves were placed in culture medium at room temperature.

Lens and ciliary body were removed and the iris was dissected from the sclera with watch-maker forceps (Dumont & Fils, Switzerland, size 5), under a stereo-dissection microscope.

To dissect sphincter and dilator, the iris was stretched on a piece of black rubber using insect pins. In such a preparation, the border between sphincter and dilator could clearly be distinguished, and they were dissected along this line with iridectomy scissors.

Culture Conditions: Immediately after dissection, the iris was transferred into Dulbecco's modified Eagle medium H21 (Gibco Laboratories, Grand Island, NY) supplemented (unless otherwise specified) with 10% rat serum, 100 U penicillin/ml, and 100 U streptomycin/ml. (Rat serum was used after preliminary experiments had demonstrated that the levels of NGF after incubation with fetal calf serum were ~20% lower than with rat serum.) Usually one iris was placed in 500 μl medium in a 24 well tissue culture cluster (Costar, Cambridge, MA) and kept at 37°C with 10% CO_2 in a water saturated atmosphere.

Determination of NGF Content in Iris and Culture Medium: NGF was determined by a two-site enzyme immunoassay which has been described in detail previously (8). Briefly, the irides were taken out of the medium, blotted on Whatman filter paper (Whatman Laboratory Products Inc., Clifton, NJ) and three to six irides were glass/glass homogenized in 500 μl 0.1 M Tris-HCl buffer, pH 7.0, containing 400 mM NaCl, 2% gelatin, 2% bovine serum albumin, and various protease inhibitors. After centrifugation at 20,000 *g* for 10 min at 4°C the supernatant was diluted 1:1 with 0.2% Triton X-100. The medium was diluted 1:1 in a 100 mM Tris-HCl buffer, containing 400 mM NaCl, 2% gelatin, 2% bovine serum albumin, 0.2% Triton X-100, and 0.1% NaN_3.

Standard curves in the range of 5 to 1,280 pg NGF/ml were determined in the buffers used for both medium and iris determinations. The detection limit (defined as the signal corresponding double blank) was 5 pg NGF/ml, which corresponded to 0.2 pg or 0.01 fmol of NGF/assay.

Previous experiments had shown that the values of rat NGF were the same when determined with affinity-purified polyclonal and monoclonal anti-mouse NGF antibodies (8). In the present experiments, taking advantage of the fact that NGF is a homodimer, we used the monoclonal anti-mouse NGF antibody 27/21 for both the first and second site of the enzyme immunoassay. The generation and properties of the antibody have been previously described (8).

Samples were incubated overnight at room temperature with the first antibody covalently linked to 1-mm diameter glass beads. The bound NGF was detected by a 2-h incubation at 37°C with the second, β-galactosidase-labeled antibody. The bound enzyme was then quantified by the generation of fluorescent 4-methylumbelliferone from 4-methylumbelliferyl-β-D-galactoside.

The mean recovery of mouse NGF added to the homogenate and to the medium was 106 ± 3% and therefore no corrections for the recoveries were made.

NGF determinations were always done in quadruplicates. Protein was determined by the method of Lowry, with bovine serum albumin as a standard (10).

The addition of the protease inhibitors leupeptin (10^{-4} M; Sigma Chemical Co., St. Louis, MO) and aprotinin (35 U/ml; Sigma Chemical Co.) for 12 h did not affect the NGF levels determined in either tissue or medium. However, the recovery of NGF secreted by the iris into the medium, decreased to 70% of initial values after a 24-h incubation period at 37°C. We have not taken this into account, because the degree to which proteases within the iris degrade NGF is not known, and thus no complete correction for overall degradation can be made.

[^{35}S]Methionine Incorporation into Protein: After 6, 12, 24, 48, or 72 h in culture, the irides were washed in serum-free medium and placed in 500 μl of this medium, containing 100 μCi/ml of [^{35}S]methionine corresponding to 7–9 × 10^7 cpm/iris (sp act 800 Ci/mmol; New England Nuclear, Boston, MA). Normal Dulbecco's modified Eagle's medium H21, which contains 200 μM L-methionine, was used for these experiments. After incubation for 1 h at the above described culture conditions, the irides were blotted on Whatman filter paper, removed, and sonicated in 100 μl 1 N NaOH. The proteins were then precipitated with 200 μl of 25% trichloroacetic acid, kept on ice for 20 min, and filtered through a Millipore filter unit (Millipore Corp., Bedford, MA), using Whatman GF/C glass microfiber filters. Each filter was washed with 10 ml 10% trichloroacetic acid followed by 5 ml ethanol and 5 ml ether. 10 ml Aqualuma scintillation liquid (J. T. Baker Chemical Co., Phillipsburg, NJ) were added to the dried filter papers and the samples were quantified by liquid scintillation spectrometry.

For the determination of the blank, irides were cultured for the corresponding time period without isotope, and then the amount of radioactivity, known to be present in the iris after a 1-h incubation with 100 μCi/ml was added directly before sonication.

Drug Treatments: Drugs were added to the medium before the irides were placed into it. Cycloheximide (Sigma Chemical Co.) was dissolved in culture medium and was present at a final concentration of 5 μg/ml. Actinomycin D (Sigma Chemical Co.), dissolved in culture medium was present at a final concentration of 10 μg/ml and 1 μg/ml and 9-β-D-arabinofuranosyladenine (ara-ade [Sigma Chemical Co.]) was dissolved in dimethylsulfoxide and was used at a final concentration of 100 μg/ml. Monensin (Sigma Chemical Co.) was dissolved in ethanol and was present at a final concentration of 10^{-6} M. The dimethylsulfoxide and ethanol containing stock solutions were diluted 1:1,000-fold in the culture medium. Drugs were present for 12 h in culture, at which time NGF levels were measured in both iris and medium.

In one set of experiments, we examined the reversibility of the effects of shorter (6 h) exposure to actinomycin D, ara-ade, or monensin. After a 6-h drug treatment, irides were washed with drug-free medium and then incubation was continued in drug-free medium for additional 6 or 18 h, at which times (12 or 24 h total time in culture) NGF levels were measured in medium and tissue. Controls were included in each experiment, incubating irides for the corresponding time periods in a drug-free medium.

RESULTS

Time Course of NGF Levels in Irides and Culture Medium

Irides were kept in culture up to 72 h and the quantities of NGF present in the irides and those released into the medium were measured after various time periods.

NGF levels in cultured irides did not differ significantly from those of freshly dissected irides for up to 3 h in vitro. Up to 12 h a very rapid increase was observed, reaching 270 ± 10 pg per iris. Thereafter, there was a slight further increase, reaching 350 ± 30 pg NGF per iris after 19 h. Subsequently, the levels fell to 220 ± 10 pg NGF at 24 h and 103 ± 22 pg NGF at 48 h. No further decrease occurred between 48 and 72 h (130 ± 10 pg NGF/iris at 72 h) (Fig. 1 *a*).

In the medium, NGF levels were not detectable until 6 h of incubation. Levels then increased to a maximal value of 1,180 ± 180 pg NGF per iris after 19 h which was maintained up to 48 h (Fig. 1 *b*). After that time the medium had to be changed, so that direct comparison with the 72-h value was not possible. However, 130 ± 20 pg NGF/iris were released between 48 and 72 h.

The most rapid rate of production of NGF, as calculated from the increase in NGF levels in the iris and the culture medium, occurred between 9 and 12 h and amounted to 203 pg NGF/h/iris.

Incorporation of [^{35}S]Methionine into Proteins

To evaluate whether the rapid decrease in NGF levels after 19 h resulted from the general deterioration of the cultured irides we determined the time course of [^{35}S]methionine incorporation into proteins after 6, 12, 24, 48, or 72 h in culture. As shown in Table I, there was no significant decrease of [^{35}S]methionine incorporation into proteins during the entire culture period, indicating that the decrease in NGF levels within the iris could not be explained by an overall decrease in protein synthesis.

Comparison between NGF Production in Sphincter and Dilator of the Iris

The iris consists of the sympathetically innervated dilator muscle and the predominantly parasympathetically inner-

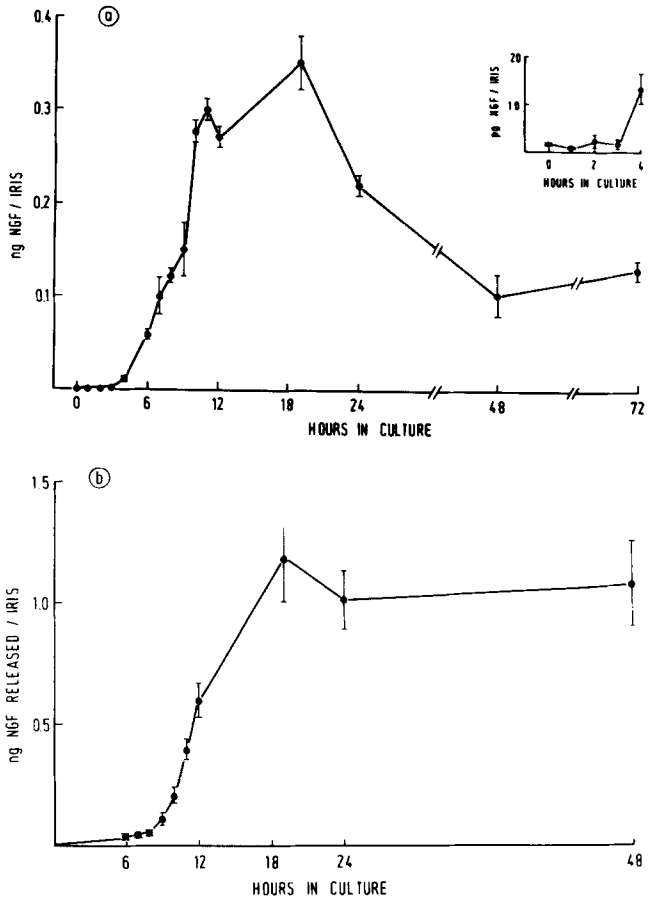

FIGURE 1 Time course of NGF levels in irides (*a*) and in culture medium (*b*). Irides were kept in culture for different time periods and NGF levels were determined as described in Materials and Methods. The values given represent mean ± SEM (n = 2–18). (*a*) Note that the values given for zero hours in culture represent in vivo NGF levels determined in irides not been brought into culture. (*Inset*) NGF levels at early time points, enlarged fivefold.

TABLE I

[^{35}S]Methionine Incorporation into Iris Protein

Hours in culture*	Amount incorporated‡ per 60 min	% of total radioactivity added
	cpm	
6	52,100 ± 3,200	0.65 ± 0.04
12	44,800 ± 1,800	0.63 ± 0.03
24	43,000 ± 500	0.60 ± 0.01
48	43,000 ± 1,700	0.54 ± 0.02
72	43,000 ± 4,300	0.51 ± 0.05

* Irides were treated as described in Materials and Methods.
‡ Blank values (1–2% of radioactivity incorporated) were subtracted. Values given represent mean ± SEM of triplicate determinations.

vated sphincter muscle, which also contains some adrenergic fibers (11). Thus we were interested to see if this would be reflected by differences in NGF levels produced by the two muscle systems, corresponding to the correlation between NGF levels and sympathetic innervation density in different target tissues (8).

Sphincter and dilator were kept separately in culture for 24 h. The NGF content of tissue and medium was measured in the same manner as that of the whole iris. The specific production of NGF in the dilator (sum of NGF present in tissue and medium per protein of tissue) was three times higher than that of the sphincter (Table II). The total amount of NGF produced by sphincter plus dilator was slightly less than that of the intact iris ($0.90 < P < 0.95$). This might be due to some inevitable tissue losses and lesions during the dissection procedure.

Level of Regulation of NGF Synthesis

To determine the level of regulation of the rapidly increasing NGF synthesis in culture, inhibitors of transcription, polyadenylation, and translation were added to the culture medium as described in Materials and Methods.

Cycloheximide treatment reduced the NGF levels in iris and medium below the detection limit of the assay (Table III). The same was true for actinomycin D at a concentration of 10 μg/ml. At a concentration of 1 μg/ml, the level of NGF was reduced to 7% of control in the medium and to 26% of control within the iris (Table III). When actinomycin D at a final concentration of 1 μg/ml was added only during the first 6 h in culture, its effect was not reversible, i.e., it reduced the NGF synthesis and release during the whole 24-h period to <10% of control values.

Ara-ade reduced the level of NGF in the medium to <6% of control and to 37% of control in the iris when it was present for the entire 12-hour period (Table III). However, when irides were exposed to ara-ade for only 6 h, the effect was partly reversible at 12 h and completely reversible at 24 h.

Intracellular Processing of NGF

The uptake of NGF by responsive neurons implies a prior secretion by the NGF-producing cells. Therefore, we tested the involvement of the Golgi apparatus, an necessary step in the pathway of secretion, in the processing of NGF by using the carboxylic ionophore monensin, which is known to interfere with the transport of proteins through the Golgi apparatus (12).

TABLE II

NGF Produced by Dilator and Sphincter of the Rat Iris during a 24-h Culture Period

	Dilator	Sphincter
NGF released into the medium	0.69 ± 0.11 ng	0.1 ± 0.01 ng
NGF remaining in the tissue	0.05 ± 0.001 ng	<0.02 ng
Protein/organ	36.9 ± 2.7 μg	14.7 ± 0.84 μg
Total NGF/mg protein of tissue	20.05 ng	6.8 ng

The values given represent mean ± SEM (n = 7 for medium, n = 2 for tissue).

TABLE III

Effect of Transcription and Translation Inhibitors

Treatment		NGF (% control)*	
		Medium	Iris
	μg/ml	%	
Cycloheximide‡	5	<3	<5
Actinomycin D‡	1	7 ± 1	26 ± 3
Actinomycin D‡	10	<6	<6
ara-ade‡	100	<6	37 ± 16

* NGF content was measured after a 12-h culture period. Mean control values see Fig. 1, *a* and *b*.
‡ Drugs were present for the whole duration of the experiment. Values given represent mean ± SEM of quadruplicate determinations.

TABLE IV

Effect of Monensin on NGF Content

	NGF (% control)*	
	Medium	Iris
	%	
Monensin‡ 0–12 h	8.3 ± 0.8	25.5 ± 8.6
Monensin§ 0–6 h	12.1 ± 5.2	62.4 ± 8.5

* NGF content was measured after a 12-h culture period. Mean control values see Fig. 1, *a* and *b*. Values given represent mean ± SEM of quadruplicate determinations.

‡ Monensin (10^{-6} M) was present for the whole culture period.

§ Irides were cultured in monensin (10^{-6} M) containing medium for the first 6 h, washed, and kept in normal culture medium for the second 6 h.

The addition of monensin to the medium for 12 h decreased NGF levels to 8% of control in the medium and within the iris itself levels were reduced to 25% of control. When monensin was present only during the first 6 h NGF levels in the medium decreased to 12% of control after 12 h, but in the tissue they were only reduced to 62% of control (Table IV). To eliminate the possibility that the monensin-mediated decrease in NGF levels is due to a general reduction in protein synthesis, we examined the effect of monensin on [^{35}S]methionine incorporation into proteins. In cultures treated with monensin for 12 h, the [^{35}S]methionine incorporation into proteins was reduced by only 20% as compared to the corresponding controls.

DISCUSSION

We studied the regulation of NGF synthesis in the cultured rat iris. When using an organ culture system, the whole amount of NGF produced can be measured, because diffusion into adjacent tissues, local degradation, and the removal by retrograde axonal transport by the innervating neurons, as well as the removal by the blood stream, can be excluded. Earlier it has been reported that various organs could produce NGF when placed in tissue culture (9, 13). However, the significance of these findings remained unclear because these results were obtained by semiquantitative bioassay techniques that could not detect the in vivo levels of NGF in intact innervated target tissues, and therefore the relation of in vivo and in vitro levels could not be established. The recent development of a sensitive enzyme immunoassay allowed the direct quantitation of endogenous NGF levels and established a correlation between NGF levels in target tissues and the density of their sympathetic innervation (8). This correlation is also reflected in our in vitro system: the densely sympathetically innervated dilator produces three times more NGF (per milligram of protein) than the sphincter that has a predominant cholinergic and a relatively sparse adrenergic innervation. From this observation we conclude that the organ specific differences in NGF synthesis are maintained in our culture system, although the NGF levels in the iris increased dramatically after culturing.

We determined the time course of NGF production both by measuring the NGF levels in tissue and the amount of NGF released into the medium. Proteolytic degradation of NGF molecules in the medium did not play an important role as far as can be judged by the unchanged NGF levels after addition of the nontoxic protease inhibitors leupeptin and aprotinin. Leupeptin is a serine and thiol protease inhibitor and aprotinin inhibits serine and various intracellular proteases (14, 15). The 30% decrease of NGF levels in medium, which was nevertheless observed after 1 d of storage at 37°C, may be due to the presence of other types of proteases. However, this decrease does not change the overall trend of the time course depicted in Fig. 1 *b* and has no influence on the other results. We found that the maximal level of 350 ± 30 pg NGF/iris after 19 h in culture was 200 times higher than in vivo. The increase of NGF levels in the iris during culture could either reflect an increase in the rate of NGF synthesis or an impaired removal by diffusion into the culture medium as compared to removal by retrograde axonal transport. However, the high amounts of NGF present in the medium argue against the latter possibility.

Additional evidence for an increased NGF synthesis in vitro can be deduced from the analysis of the time course of the rate of synthesis during the culture period. The increase in the rate of synthesis between 6 and 12 h strongly supports a stimulation of NGF synthesis in that time period.

The maximal NGF levels in the iris were reached after 19 h in culture. Subsequently, the NGF levels decreased to one third of the maximal level at 48 h and then remained stable up to 72 h. This decrease is specific, since it is not caused by a general deterioration of protein synthesis, shown by a constant rate of [^{35}S]methionine incorporation into proteins during the whole culture period (Table I).

In a recent study the level of NGF in the rat iris in vivo was estimated by a two-site radioimmunoassay (16). The values amounted to 5–10 pg NGF/iris which is three- to sixfold higher than our value. However, this discrepancy may originate from the fact that the NGF level was at the detection limit of their assay method, i.e., below the lowest NGF concentration measured in their standard curve. Ebendal et al. (9) also found with bioassay technique an increase of NGF levels after tissue culture for 24 and 48 h, to values which were much lower than the NGF levels we have determined. This discrepancy, however, may result from different culture conditions (collagen gels as substrate instead of free floating irides).

The mechanisms responsible for the early enhancement and the later decrease of NGF synthesis in culture are unclear at present. We found no evidence for an effect specific to rat serum, since similar results were obtained with fetal calf serum. The time period of NGF increase in tissue culture roughly corresponds to the time period of degeneration of the nerve terminals after axotomy (17, 18). It may be that during their degeneration an inducing factor is released, and that the enhanced formation of NGF can be interpreted as part of a regeneration mechanism in vivo. The subsequent decrease in NGF levels might then be due to a limited life time of the postulated inducing factor, or to a negative feedback mechanism. In any event, the NGF level in the iris remained stable between 48 and 72 h at a 60-fold higher level than in vivo. The persistantly elevated level could reflect a repression of NGF synthesis by the intact nerve terminals in vivo. It remains to be established whether this postulated repression is due to cell-cell contact between neurons and target cells or to a soluble factor released by nerve terminals.

To study the mechanism of NGF synthesis, we examined the effect of various drugs interfering with mRNA and protein synthesis. The results showed that the transcription inhibitor actinomycin D, the polyadenylation inhibitor ara-ade (19), and the translation inhibitor cycloheximide, all were able to

block NGF synthesis. While these data may imply that the increased NGF synthesis in culture results from an augmented NGF-mRNA synthesis, it cannot be excluded that these inhibitors interfere with the synthesis of molecules involved in the processing of a large stock of NGF precursor. These two possibilities can only be distinguished by quantitation of NGF-mRNA.

Further studies have been performed with monensin, which interferes with the transfer of peptides through the Golgi apparatus (12). We conclude, from the decrease of NGF levels in the medium after monensin treatment, that the Golgi apparatus is involved in the intracellular processing of NGF. The concomitant decrease of NGF levels within the iris was somewhat unexpected. One possible explanation for this observation could be that monensin blocks the transport of the protein proximal to the site of proteolytic processing of the NGF precursor (20). The monoclonal antibodies raised against mature NGF, which were used in this study probably do not recognize the precursor molecule, as has been shown for polyclonal antibodies (21).

In preliminary experiments, we found that removal of Ca^{++} (with EGTA or EGTA and the calcium ionophore A23187) had no inhibitory effect on the release of NGF (Barth, E.-M., unpublished results). This indicates that NGF is secreted by the constitutive pathway of secretion (22) in contrast to the classical pathway of stimulus induced secretion from storage vesicles.

In conclusion, we have shown that the NGF synthesis is largely stimulated in culture and that transcriptional processes are involved in the enhancement of NGF synthesis. It remains to be investigated what is responsible for this stimulation and furthermore which cell type(s) is synthesizing NGF. At the moment it cannot be decided whether NGF is produced by smooth muscle cells, fibroblasts or by glial cells.

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