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

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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 released 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 systems (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 determi-

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1 Abbreviations used in this paper: ara-ade, 9-β-D-arabinofuranosyladenine; NGF, nerve growth factor.
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 watchmaker forceps (Dumont & Fils, Switzerland, size 5), under a stereomicroscope.

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% CO2 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 µ1 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% NaN3.

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 0.02 pg or 0.01 fmol of NGF/assay.

Previous experiments had shown that the recoveries 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 and washed. The second antibody was then coupled with 125I using the chloramine-T method. The bound enzyme was then quantified by the generation of fluorescence. 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.

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RESULTS

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 230 ± 10 pg NGF/iris 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 [35S]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 [35S]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 [35S]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-
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**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Amount incorporated*</th>
<th>% of total radioactivity added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1500 ± 300 cpm</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>3000 ± 600 cpm</td>
<td>0.30 ± 0.02</td>
</tr>
</tbody>
</table>

* Irids were treated as described in Materials and Methods.

**TABLE III**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Medium</th>
<th>Iris</th>
</tr>
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<tbody>
<tr>
<td>Cycloheximide</td>
<td>5 μg/ml</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>1 μg/ml</td>
<td>7 ± 1%</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>10 μg/ml</td>
<td>26 ± 3%</td>
</tr>
<tr>
<td>ara-ade</td>
<td>100 μg/ml</td>
<td>37 ± 16%</td>
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</tbody>
</table>

* NGF content was measured after a 12-h culture period. Mean control values see Fig. 1, a and b.

**TABLE IV**

<table>
<thead>
<tr>
<th>Effect of Transcription and Translation Inhibitors</th>
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<tbody>
<tr>
<td>Treatment</td>
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<tr>
<td>-----------</td>
</tr>
<tr>
<td>Cycloheximide</td>
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<td>Actinomycin D</td>
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<td>Actinomycin D</td>
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* Drugs were present for the whole duration of the experiment. Values given represent mean ± SEM of quadruplicate determinations.
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 than that which has a predominantly 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. 1b 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 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
Nerve Growth Factor Synthesis and Release in Rat Iris

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