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Catecholamines increase nerve growth factor mRNA content in both mouse astroglial cells and fibroblast cells

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Previous studies have shown that catecholamines increase the nerve growth factor (NGF) content in medium conditioned by mouse L-M fibroblast cells and mouse astroglial cells. In this study, the NGF mRNA levels in these cells were measured by Northern blot analysis. In astroglial cells treated with epinephrine (EN), the cellular NGF mRNA level increased prior to accumulation of NGF in the culture medium. 3-Hydroxytyramine (DA) and norepinephrine (NE) also increased the cellular NGF mRNA content. An increased level of NGF mRNA elicited by EN was also observed in mouse L-M cells. These results indicate that catecholamines enhance NGF synthesis of L-M fibroblast cells and astroglial cells by increasing the cellular content of NGF mRNA. The present results also indicate that the effects of catecholamines are not mediated by adrenergic receptors.

Nerve growth factor; Catecholamine; Inducer; Methylcatechol, 4-; (Fibroblast, Astroglial cell)

1. INTRODUCTION

Nerve growth factor (NGF) was initially described as a protein required for development and maintenance of sympathetic and sensory neurons in the peripheral nervous system (PNS) [1]. Recent studies have also suggested important functions for NGF in the cholinergic neurons of the basal forebrain in the central nervous system (CNS) [2]. In previous reports, we showed that fibroblast cells cultured from mouse organs with sympathetic innervation [3,4], an established cell line of mouse fibroblast cells [5], and astroglial cells cultured from mouse brain [6] synthesize and secrete NGF that is indistinguishable from mouse submaxillary gland β-NGF. Catecholamines cause a significant increase in the NGF content of the conditioned medium (CM) of fibroblast cells [5,7] and of astroglial cells in the quiescent phase [8]. Catecholamines do not affect viability of the cells, or their level of total protein synthesis [5,8]. The possibility that catecholamines suppress NGF degradation or stimulate NGF secretion may also be ruled out [5,8]. The accumulated data would suggest that catecholamines stimulate the de novo synthesis and secretion of NGF protein. In order to examine the de novo synthesis, Northern blot analysis of NGF mRNA was performed in this paper.

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Abbreviations: NGF, nerve growth factor; EN, epinephrine; NE, norepinephrine; DA, 3-hydroxytyramine; PNS, peripheral nervous system; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; CM, conditioned medium; EIA, enzyme immunoassay; BSA, bovine serum albumin

2. MATERIALS AND METHODS

2.1. *Materials*

Mouse submaxillary gland β-NGF and anti-β-NGF antiserum

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were prepared as in [9]. DMEM, FCS, and trypsin were obtained from Gibco; Medium 199, from Flow Labs; peptone, from Difco; tissue culture vessels, from Falcon; BSA, from Armour; (−)-EN, DA, L-phenylephrine and salbutamol, from Sigma; (−)-NE, from Nakarai; 4-methylcatechol, from Tokyo Kasei; [γ-^{32}P]ATP (>5000 Ci/mmol), [α-^{32}P]dCTP (~3000 Ci/mmol) and multipriming labeling kit, from Amersham; AMV reverse transcriptase, from Life Science; dATP, dCTP, dGTP, TTP, and oligo(dT)-cellulose type 7, from Pharmacia; nick-translation kit, from Bethesda Research Labs; T_4 polynucleotide kinase and restriction enzymes, from Takara Shuzo; actin and c-*myc* probes, from Oncor; and nitrocellulose paper (0.45 μm, BA85), from Schleicher & Schuell. All other chemicals were reagent grade.

2.2. *Cell culture*

Mouse astroglial cells were cultured from the whole brain of newborn mice and maintained in DMEM containing 10% FCS as described [6]. The meninges of the brain were carefully removed. Cells were put into the quiescent phase by serum deprivation [10]. Mouse L-M cells were obtained from the American Type Culture Collection and maintained as monolayer cultures in Medium 199 supplemented with 0.5% peptone as in [5].

2.3. *Preparation of hybridization probes*

Poly(A)$^+$ RNA was isolated from the submaxillary glands of 65-day-old male ICR mice according to Cathala et al. [11]. A cDNA of length 341 bp covering almost the entire mouse β-NGF mature peptide was obtained from the poly(A)$^+$ RNA, and the hybridization probe to detect NGF mRNA was prepared as described below. A synthetic oligonucleotide 30-mer, which was complementary to the sequences between amino acid residue 107 and 116 [12], was used to prime specifically reverse transcription of the isolated submaxillary gland poly(A)$^+$ RNA and a cDNA library was constructed [13]. The 27-mer which was complementary to the sequences between amino acid residues 28 and 36 [12] and the 30-mer mentioned above were kinased with [γ-^{32}P]ATP and T_4 polynucleotide kinase, and then used to screen the cDNA library [14]. A 341 bp long cDNA fragment, whose nucleotide sequence was in accord with nucleotides 641–982 (amino acid residues 3–116) of the reported mouse NGF sequence [12], was isolated and used to prepare the hybridization probe. A ^{32}P-labeled single-stranded DNA was prepared by annealing the cloned 341 bp cDNA with the 30-mer and polymerizing an antisequence DNA with [α-^{32}P]dCTP, dATP, dGTP, TTP and Klenow enzyme. Use of the ^{32}P-labeled probe thus obtained (primer extension method; spec. act. 2.8–7.1 $\times$ 10^8 cmp/μg) resulted in a higher intensity of the hybridization image as compared with the probe labeled using the Amersham multipriming labeling system (random priming method; spec. act. 1.2 $\times$ 10^9 cpm/μg). Commercially obtained chicken actin DNA (770 bp) and human c-*myc* 3rd exon DNA (1800 bp) were nick-translated [15] to a specific activity of 4.5 $\times$ 10^8 and 6.6 $\times$ 10^7 cpm/μg, respectively, and used to detect the levels of actin mRNA or c-*myc* mRNA.

2.4. *Determination of NGF mRNA level*

For the determination of NGF mRNA, poly(A)$^+$ RNAs were prepared [16] from cultured cells (about 2 $\times$ 10^8 cells). Poly(A)$^+$ RNAs treated with 50% formamide were separated on 1% agarose-formaldehyde gels, and then transferred onto nitrocellulose filters. Hybridization with the ^{32}P-labeled probe (approx. 4 $\times$ 10^6 cpm/ml) was performed with 50% formamide at 42°C for 24 h. After stringent washing, the filters were autoradiographed at −80°C for 1–3 days with Kodak intensifying screens. The autoradiogram was scanned at a path width of 0.8 mm (*X*-axis) with an LKB 2222 densitometer.

2.5. *Determination of NGF level*

For determination of NGF, the CM was directly applied to the EIA system [3,6]. The detection limit of the EIA with submaxillary gland NGF was 5 pg/ml [9]. The EIA system was not affected by the drugs or culture media used in this work.

3. RESULTS

As shown in fig.1, mouse L-M cells produced a moderately abundant quantity of NGF mRNA that was easily detectable in as little as 1 μg of total cellular poly(A)$^+$ RNA. This mRNA was indistinguishable from authentic NGF mRNA extracted from mouse submaxillary gland regarding hybridization behavior and electrophoresis through agarose gels. The detection limit of this assay with submaxillary gland poly(A)$^+$ RNA was 0.1 ng/lane. The effect of 0.1 mM EN on the NGF mRNA level in mouse L-M cells was examined after 6 h exposure to EN, since EN is known to increase the NGF content of the CM after a lag time of 4 h, which remains elevated for 24 h [5]. As shown in fig.1, the NGF mRNA level after 6 h was 2.4-fold greater than the control level.

Northern blot analysis on mouse astroglial cells showed the expression of a major 1.3 kb transcript which was identical in size to mouse submaxillary gland β NGF mRNA (fig.2A). The effect of catecholaminases (0.15 mM EN, NE and DA) on the NGF mRNA level in astroglial cells was assessed after a few hours of incubation. EN and DA increased the NGF mRNA levels 5–7-fold (figs 2A,3), the rise with NE being about 1.5-fold (not shown). These results agree with previous findings that EN and DA obviously increased the NGF content in CM of astroglial cells, whereas NE did so but only slightly [9].

A time-course experiment revealed that the NGF content in the medium of control cultures increased in proportion to culture time, and the addition of 0.15 mM EN accelerated this increase in NGF content after a lag time of 4 h (fig.2B). Intracellular NGF contents were low and remained unchanged during the course of the experiment (not shown).

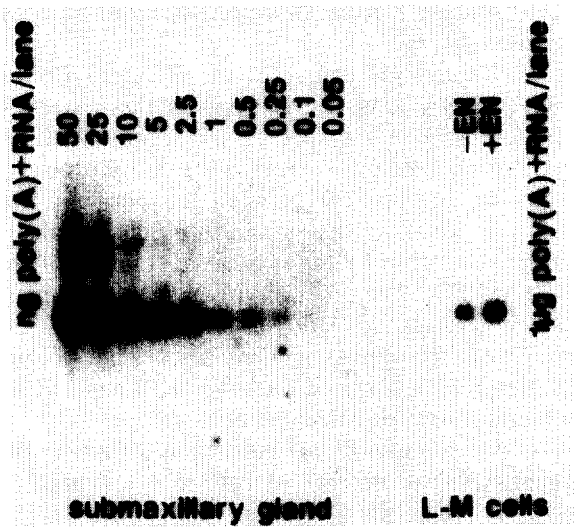

Fig.1. Northern blot analysis of poly(A)$^+$ RNA from mouse submaxillary gland and mouse L-M cells. L-M cells were cultured in the presence or absence of 0.1 mM EN for 6 h. Poly(A)$^+$ RNA (0.05–50 ng) extracted from mouse submaxillary gland and 1 μg L-M cell poly(A)$^+$ RNA were analyzed by Northern blot hybridization as described in section 2.

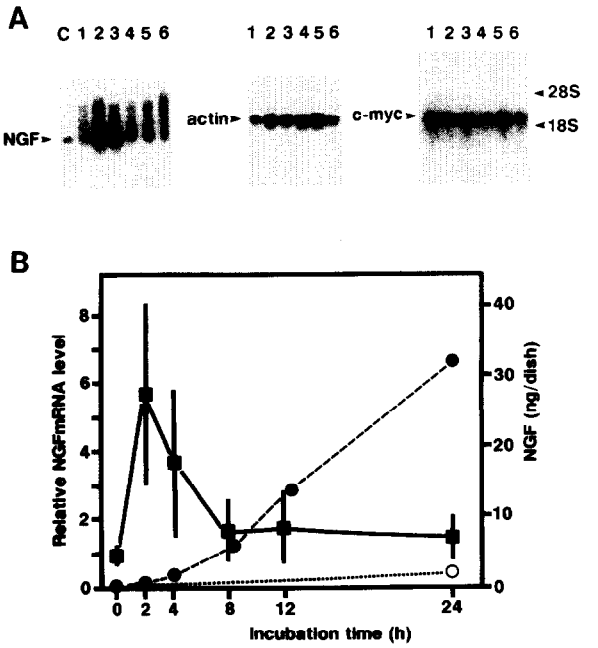

Fig.2. Time course of the effects of 0.15 mM EN on cellular NGF mRNA and NGF levels in the medium of mouse astroglial cells. (A) Poly(A)$^+$ RNA (1.5 μg) from astroglial cells treated with 0.15 mM EN for 0, 2, 4, 8, 12 and 24 h (lanes 1–6, respectively) and 1 ng mouse submaxillary gland poly(A)$^+$ RNA (lane C) were hybridized to ^{32}P-labeled NGF, actin, and c-*myc* cDNA probes. The filters were autoradiographed at −80°C for 20 min (for determination of actin mRNA) of 2 days (for assay of NGF mRNA and c-*myc* mRNA). (B) NGF mRNA levels (■) are expressed as fold increase over that in the absence of EN (mean ± SE of 3 experiments). NGF levels with (●) or without EN (○) are expressed as ng per 100 mm dish.

The NGF mRNA level in EN-treated astroglial cells increased 5.7-fold in 2 h, dropping to 1.7-fold over the normal level in 8 h (fig.2A,B). Equal amounts of poly(A)$^+$ RNA from each sample were then assayed for actin mRNA and c-*myc* mRNA. Fig.2A shows that actin mRNA and c-*myc* mRNA were detected at nearly constant levels over the duration of the experiment.

We previously suggested that the stimulatory effects of catecholamines on NGF synthesis are due to the catechol part of the molecule [5,7], and are not mediated by adrenergic receptors [5]. In order to assess this possibility, we measured NGF mRNA levels after exposure of astroglial cells to phenylephrine, an α-agonist or to salbutamol, a β-agonist, which do not contain the catechol ring. As shown in fig.3, neither phenylephrine nor salbutamol increased the NGF mRNA level. We also investigated the effect of 4-methylcatechol, a non-amine catechol, on the NGF mRNA level. Fig.4 shows that treatment of L-M cells with 0.15 mM 4-methylcatechol for 4 h increased the NGF mRNA level 2.1-fold. 4-Methylcatechol also increased the NGF mRNA level of astroglial cells 8.1-fold.

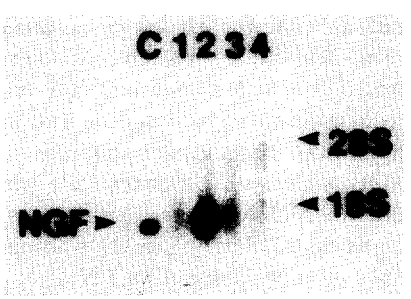

Fig.3. Effects of adrenergic agonists on the level of NGF mRNA in mouse astroglial cells. Poly(A)$^+$ RNA (1.5 μg) from untreated astroglial cells (lane 1) or from those treated with 0.15 mM DA (lane 2), phenylephrine (lane 3), or salbutamol (lane 4) for 3.5 h was analyzed by Northern blot analysis with ^{32}P-labeled NGF cDNA. Poly(A)$^+$ RNA (1 ng) from mouse submaxillary gland was also analyzed (lane C).

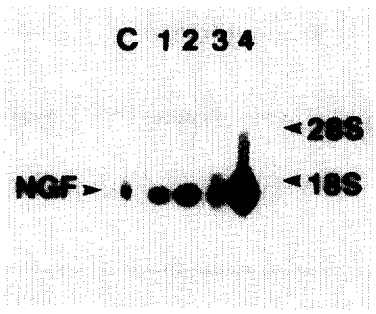

Fig.4. Effects of non-amine catechol on the level of NGF mRNA in mouse L-M and astroglial cells. Cells were treated with 0.15 mM 4-methylcatechol for 4 h. Samples (1.5 μg) of poly(A)$^+$ RNA from control L-M cells (lane 1), 4-methylcatechol-treated L-M cells (lane 2), control mouse astroglial cells (lane 3), and treated astroglial cells (lane 4) were electrophoresed, transferred to nitrocellulose and hybridized with a ^{32}P-labeled NGF probe. Poly(A)$^+$ RNA (1 ng) from mouse submaxillary gland was also analyzed (lane C).

4. DISCUSSION

The present paper extends our previous reports that catecholamines markedly increase the NGF content in both fibroblast cells [5] and astroglial cells [8]. The present data reveal that catecholamines increase the cellular concentration of NGF mRNA in both cells; namely, catecholamines increase the NGF concentration in CM primarily by increasing the cellular content of NGF mRNA. To assess the specificity of the effect of catecholamines on the level of NGF mRNA, we determined actin mRNA and c-*myc* mRNA levels after exposure of astroglial cells to EN. The results in fig.2A indicate that the effects of catecholamines are specific for NGF mRNA. We previously demonstrated that catecholamines did not stimulate incorporation of [^{3}H]thymidine into astroglial cells, suggesting that catecholamines did not elevate NGF synthesis by initiating cell growth [8]. As the c-*myc* mRNA level is known to be cell-growth-dependent [17], the present findings that c-*myc* levels were not changed by EN are in accord with the previous suggestion.

Our observations that neither phenylephrine nor salbutamol increased the NGF mRNA level whereas 4-methylcatechol did (figs 3,4) support our previous suggestion that the catechol part of the catecholamine molecule is necessary and that adrenergic receptors of cells do not participate in the stimulatory effect of catecholamines on NGF synthesis [5,7].

The rapid time course of the effect of EN on NGF mRNA level (fig.2) suggests that catecholamines increase the rate of transcription of the NGF gene [18,19]. Further work is necessary in order to differentiate between the possibility of transcriptional activation and that of posttranslational regulation by an increase in stability of the NGF mRNA. The question arises as to how many catecholamines activate the transcription of the NGF gene. To approach an answer, we have begun to identify the sequences of the NGF gene which are important for catecholamine action.

It is of interest to determine whether the phenomena we observe in culture are evoked in vivo. It seems plausible that NE contributes, at least in part, to the regulation of the NGF mRNA level in the PNS, since (i) there is a positive correlation between the NGF mRNA level and NE content in the effector organs of sympathetic nerves [20]; (ii) the concentration of NE in the vicinity of sympathetic nerve terminals was estimated to be of the order of 10000 μg/g [5]. We have begun to examine the NGF and NGF mRNA levels in the PNS and CNS after administration of catecholamine analogues.

It is also interesting to ascertain whether a catecholamine-sensitive sequence of the NGF gene exists in human genomic DNA. Clarification of this possibility will lead to a better understanding of the regulatory mechanism of NGF gene expression and the pathogenesis of various degenerative nervous diseases, such as Alzheimer's disease. Enhancement of the synthesis of endogeneous NGF by catecholamine analogues may become a promising therapy for various nervous diseases in the future.

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