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Enhancement of Neural Death by Nerve Growth Factor

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Abstract: Nerve growth factor (NGF) is literally known to promote neural differentiation and survival in several peripheral and central neurons. Thus, it is widely believed that NGF may serve as a therapeutic agent for many types of neuronal diseases. One of the mechanisms suggested to explain the protective role of NGF is that the trophic factor can prevent the increase of intracellular calcium ions which might be responsible for neural death. To examine whether or not the calcium hypothesis works even under pathological conditions, we applied NGF to cultures deprived of glucose. Surprisingly, what was observed here is that NGF rather promoted cell death under a glucose-deprived condition. What we call the NGF paradox phenomenon occurred in a calcium concentration-dependent manner, indirectly suggesting that NGF might increase intracellular calcium ions in cells deprived of glucose. This suggestion is further supported by the fact that nifedipine, a well-known L-type calcium channel blocker, could block the cell death potentiated by NGF. Here it is still premature to propose the complete mechanism underlying the NGF paradox phenomenon. However, this study certainly indicates that NGF as a therapeutic agent for neuronal diseases should be carefully considered before use.
Key words: cell death, glucose-deprivation, neurotrophins, nerve growth factor paradox, PC12 cells.

Nerve growth factor (NGF), of the neurotrophin family, is a target-derived, trophic protein which targets for a variety of neurons such as peripheral sympathetic neurons and central neurons (Korsching and Thoenen, 1983; Shelton and Reichart, 1984; see a review article, Levi-Montalcini, 1987). It promotes neurite outgrowth of sensory neurons in chick embryos (Levi-Montalcini and Angeletti, 1968) and increases neuronal survival during development (Hamburger *et al.*, 1981).

In addition to its role as a key factor in neural differentiation and developemnt, NGF is known to have a neuroprotective role against several insults. Hefti (1986) found that NGF promotes survival of septal cholinergic neurons after fimbrial transections. Kromer (1987) showed that the intraventricular administration of NGF can reduce the retrograde neuronal death of septal cholinergic neurons after axotomy. NGF also decreases hippocampal degeneration resulting from ischemic injury (Shigeno *et al.*, 1991). The possible role of NGF as a neuroprotective factor implied by these *in vivo* results is further supported by the following *in vitro* studies. When sympathetic neurons grown in culture are deprived of NGF, their neurites become fragmented, cell bodies become condensed, and ultimately cell death results (Martin *et al.*, 1988). In cultured rat hippocampal and human cortical neurons, NGF decreases neural death against glucose deprivation- and iron-induced injury (Cheng and Mattson, 1991; Zhang *et al.*, 1993). All these studies suggest that NGF may be useful as a therapeutic agent for both acute and chronic neurodegenerative diseases.

Although Mattson and his collegues (Mattson *et al.*, 1991a & b; Cheng *et al.*, 1993) recently suggest that intracellular calcium alterations induced by NGF may be responsible for the protective activity of NGF, the underlying biochemical mechanism for the neuroprotective activity of NGF has not yet been fully understood. Much biochemical evidence about the functions of NGF has been accumulated using rat pheochromocytoma cells (PC12). For example, in PC12 cells NGF increases the density of sodium channels (Kirschenbaum and Greene, 1982), induces synthesis of neurotransmitter-synthesizing enzymes (Feinstein *et al.*, 1985), changes pharmacological properties of voltage-sensitive calcium channels (Takahashi *et al.*, 1985), induces the genes for calcium-binding proteins (Masiakowski and Shooter, 1988), and triggers the activation and/or tyrosine phosphorylation of a series of intracellular proteins such as PLC-γ_1 (Kim *et al.*, 1991), $p21^{ras}$ (Qiu and Greene, 1991), B-Raf (Oshima *et al.*, 1991), MAP kinases (Boulton *et al.*, 1991), phosphatidylinositol 3-kinases (Soltoff *et al.*, 1992), and SHC (Pelicci *et al.*,

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1992). Therefore, we have chosen PC12 cells as a working model for the elucidation of the biochemical relation of intracellular calciums and NGF in a pathological state. Here we report that NGF potentiated cell death when it was applied to PC12 cells in a pathological state such as glucose-deprivation. On the other hand, it apparently rescued cells when it was applied prior to glucose-deprivation.

Materials and Methods

Cell culture and pharmacology

Rat pheochromocytoma PC12 cells were grown at 37℃ in RPMI-1640 medium (growth media, GM; Gibco, Grand Island, USA) supplemented with 10% horse serum (Gibco, Grand Island, USA), 5% fetal bovine serum (Gibco, Grand Island, USA), and 0.01% PSN antibiotic mixtures (Gibco) in a humidified atmosphere of 90% air and 10% CO_2 in the absence of NGF. The cultures used for each experiment were prepared by replating growing PC12 cells in 96-well culture dishes (Falcon Labware, Oxnard, USA) coated with 0.01% poly-L-lysine (Sigma Chemical Co., St. Louis, USA). The replating density of PC12 cells was of 40,000~60,000 cells per well (sample volume of each well, 100 μl).

Two days after replating, cells were exposed to a glucose-withdrawl insult by replacing GM with a glucose-deficient medium, a balanced salt solution (BSS). Cultures were washed by aspirating GM in each well down to 20 μl with a 8-channel pipetter and adding 200 μl of BSS, which was repeated 3 times. Then 80 μl of BSS was added, making a sample volume of each well 100 μl. For control experiments, BSS including 6 mM glucose were used. BSS contained (in mM): 124 NaCl, 5 KCl,1 $MgSO_4$, 1 NaH_2PO_4, 2 $CaCl_2$, 26 $NaHCO_3$, 0.05% phenol red; had a pH of 7.4. Its osmolarity was 300 mosmol. Ten μl of NGF (7s from mouse submaxillary gland; Sigma) was added to each well of culture 2 days before and 30 min after the onset of glucose-deprivation for the pre-treated and post-treated experiments, respectively.

For the calcium experiments, BSS variants were used where $CaCl_2$ in BSS was replaced with choline chloride. Also sister cultures were prepared in the absence of NGF. The stock solutions of nifedipine (0.1 M), purchased from Sigma Chemical Co. (St. Louis, USA) were prepared in dimethyl sulfoxide (DMSO). The working nifedipine solution, added to each well by 10 μl, was prepared by diluting with the vehicle solution of BSS containing 0.01% DMSO so that the final concentration of DMSO in each well might be 0.001% (v/v). DMSO (0.001%) itself did not show any effect on cell survival (data not shown).

Measurement of cell survival

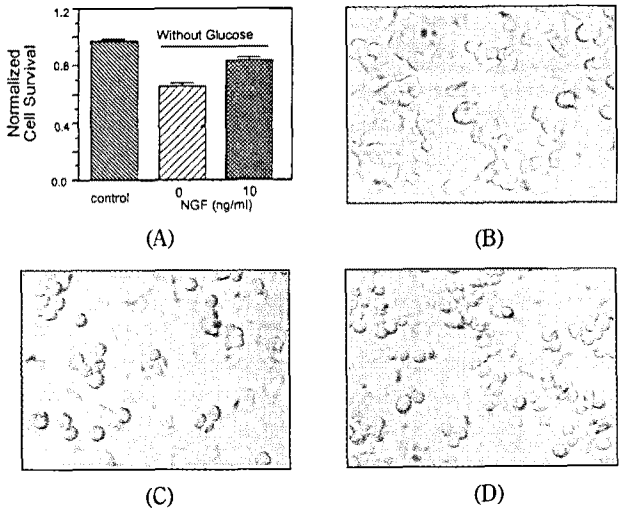

Fig. 1. The effect of glucose-deprivation on neuronal survival. PC12 cells were exposed to a glucose-deprived condition 2 days after the incubation in GM with or without 10 ng/ml NGF. (A) NGF applied prior to glucose-deprivation reduced neural death caused by glucose-withdrawl. Cell survivals for cultures deprived of glucose were normalized with respect to those for control cultures in which GM were replaced with balanced salt solutions (BSS) including glucose. No NGF was added to control cultures. *Normalized data obtained from mean values (N=8); Error bars, SEM.* (B) PC12 cells in control. Cell shape and short processes were relatively well maintained even in the BSS including glucose. (C) PC12 cells in glucose-deprivation. Cell density was dramatically reduced and cells were rounded-up without showing any processes. (D) NGF-pretreated PC12 cells in glucose-deprivation. Most of cells were rounded-up, but the extent of cell loss was significantly decreased with respect to the cultures without NGF. *Magnification ratio×320 for (B), (C) and (D).*

Twenty hours after the addition of NGF, the effect of NGF on cell survival in a glucose-deprived condition was examined using a colorimetric assay, with minor modification, described by Hansen and his colleagues (1989). Briefly, BSS with glucose replaced BSS just before the start of the MTT assay in order to stop the glucose-deprivation. Then, 2 mM of 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium (MTT) was added to each sample. Two hours after incubation with MTT at 37℃ with 10% CO_2, a lysing buffer (20% of sodium dodecyl sulfate in 50% N,N-dimethyl formamide; pH adjusted to 4.7) was added. After an overnight incubation at 37℃, the absorbance of formazans extracted in each sample was determined at 570 nm using an ELISA reader (Molecular Devices, Menlo Park, USA).

Results and Discussion

Glucose-deprivation

The population doubling-time of PC12 cells in our culture conditions was 2 to 3 days. Since cell behavior and biochemistry generally change significantly at each phase of cell growth, PC12 cells in log-phase of the

growth cycle, 2 days after seeding, were subject to pharmacological experiments in this study. One day after PC12 cells were exposed to glucose-deficient media, cells were significantly ($p<0.01$, $N=8$) degenerated by 32% with respect to controls where BSS containing 6 mM glucose replaced GM (Fig. 1A). The extent of degeneration of PC12 cells by glucose-withdrawl was much less than that of rat hippocampal neurons (Cheng *et al.*, 1993), which may be due to the different biochemical properties of cell-line cultures vs. primary cultures and/or peripheral neurons vs. central neurons. Representative was the disappearance of small processes and shrinkage of cells with a lowered cell density in glucose-deprived cultures (Fig. 1C). The pretreated NGF is known to completely block the degeneration of hippocampal neurons against hypoglycemia (Cheng *et al.*, 1993), which is not exactly the case for PC12 cells in this study. Even when 10 ng/ml of NGF were added to cultures 48 h prior to the onset of hypoglycemia, cell degeneration still occurred under the glucose-deprived condition (Fig. 1D). However, the extent of cell death was significantly ($p<0.01$, $N=8$) lower than that in cultures without NGF (Fig. 1A). This result is, therefore, still comparable to the previous findings (Cheng and Mattson, 1991; Cheng *et al.*, 1993) in that the pretreated NGF shows a neuroprotective effect on cultured neurons against hypoglycemic damage.

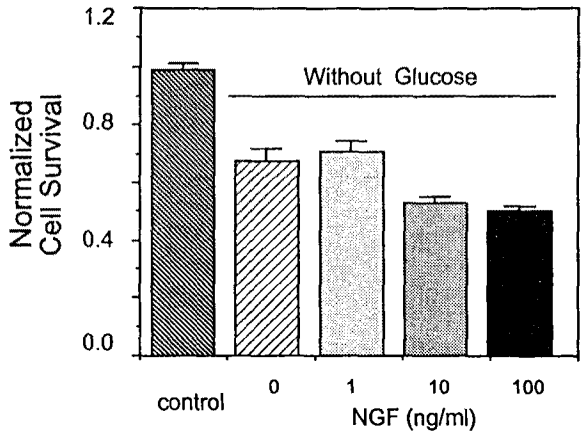

Fig. 2. NGF-induced neural death. NGF potentiated neural death caused by glucose-withdrawl in a concentration-dependent manner, when it applied to the cultures in glucose-deprivation. One ng/ml NGF showed no significant effect on either neuronal survival or death while 10 and 100 ng/ml NGF potentiated neuronal death caused by glucose-deprivation. Note that pretreated NGF (10 ng/ml) apparently reduced neural death caused by glucose-deprivation (see Fig. 1). Cell survivals for each culture in BSS (glucose-free) were normalized with respect to those for control cultures maintained in BSS including glucose. *Normalized data obtained from mean values (N=16); Error bars, SEM.*

The potentiation of cell death by NGF

Neuronal loss in hypoxic/ischemic injury could be blocked by antagonists of the N-methyl-D-aspartate receptor, which activates channels characterized by high calcium permeability (Koh and Choi, 1987). Also, intracellular calcium levels increased in cultured cortical neurons deprived of oxygen and glucose (Goldberg and Choi, 1990). These studies indicate that neural injury may be mediated by the increase of intracellular calcium. NGF is also known to increase intracellular calcium levels by activating L-type calcium channels in sympathetic and dorsal root ganglion neurons (Koike *et al.*, 1989; Rich and Hollowell, 1990; Johnson *et al.*, 1992). We therefore made a hypothesis on the "NGF paradox" that NGF may accelerate cell death by increasing further intracellular calcium levels of neurons which are already in a hypoglycemic condition. In order to examine this hypothesis, NGF was applied to the culture after the onset of glucose-deprivation.

As expected, NGF potentiated cell death when it was applied to cultures 30 min after the onset of hypoglycemia (Fig. 2). It occurred in a concentration-dependent manner. No effect of NGF on cell viability was observed when 1 ng/ml of NGF was used. Application of 1 ng/ml NGF potentiated cell death by 29% which was not significantly different from the potentiation of cell death (32%) in cultures without NGF. However, 10 and 100 ng/ml of NGF potentiated cell death by 47 and 50%, respectively. Accordingly, 32% of cells were more significantly ($p<0.01$, $N=16$) degenerated in cultures with 10 ng/ml of NGF than those without NGF. The acceleration of cell death seemed likely to be saturated at 100 ng/ml of NGF since no significant differences were observed in the cell death potentiated by 10 and 100 ng/ml NGF. This result was rather surprising, even though we expected it, because NGF was found to protect central neurons against hypoglycemic damage even when applied after the onset of hypoglycemia (Cheng and Mattson, 1991). It is noteworthy that NGF was recently found to have neither protective nor potentiating effect on cortical neurons against apoptotic and necrotic injury, respectively (Koh *et al.*, 1995). Interestingly, other neurotrophic factors such as brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5 potentiated necrotic damage of cortical neurons (Koh *et al.*, 1995). It is thus possible that the action of growth factor may differ depending upon the target cell type; that is to say, NGF potentiates degeneration for peripheral neurons not for central neurons while BDNF does it in a reverse manner.

Calcium-dependence of NGF action

Our hypothesis is based upon the assumption that

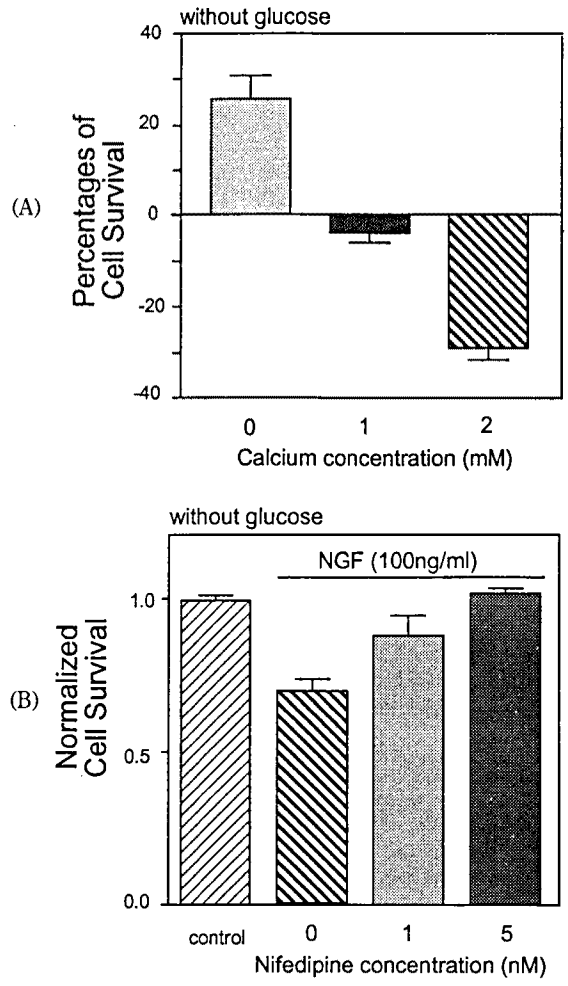

Fig. 3. Calcium-dependence of NGF action in a glucose-deprived condition. (A) Effect of extracellular calcium on NGF-induced neural death in glucose-deprivation. The effect of NGF (100 ng/ml) on cell survival and death was apparently dependent on extracellular calcium. In the absence of extracellular calcium NGF prevented cell death induced by glucose-deprivation whereas in the presence of extracellular calcium it potentiated cell death. The higher the extracellular calcium, the more neurons were subject to death. Note that the relative data (%) used here were obtained from comparing the cultures subject to NGF treatment with the sister cultures without NGF. *Error bars, SEM.* (B) Effect of nifedipine on NGF-induced neural death in glucose-deprivation. The neuroprotective effect of nifedipine, a calcium channel blocker, occurred in a concentration-dependent manner. NGF-induced cell death was prevented by 60 and 100%, respectively, with 1 and 5 nM nifedipine. Cell survivals for each culture exposed to nifedipine and NGF (100 ng/ml) were normalized with respect to those for control cultures exposed only to vehicle solutions. *Normalized data obtained from mean values (N=4); Error bars, SEM.*

the increase of intracellular calcium plays an important role in neural death. To examine indirectly whether or not the potentiation of cell death by NGF in hypoglycemic cultures depends on the intracellular calcium concentration, the extracellular calcium concentration in BSS was altered. The results are very interesting (Fig. 3A). When extracellular calcium was omitted, 25% more of cells were survived in cultures with NGF (100 ng/ml) than in sister cultures without NGF. On the other hand, 4 and 29% of the cells degenerated, respectively, more in 1 and 2 mM calcium-saline-cultures with NGF than in the corresponding sister cultures without NGF. The fact that cell death occurred significantly ($p< 0.001$, $N=4$) more in 2 mM than in 1 mM calcium-saline-cultures suggests that NGF-induced cell death could be dependent on the concentrations of extracellular calcium. One possible explanation for this differential action of NGF depending upon extracellular calcium is this; NGF applied to the cultures without extracellular calcium would activate transmembrane calcium channels through which calcium outflux might occur simply depending on the calcium concentration gradient across the membrane. Then, intracellular calcium levels enhanced by glucose-deprivation would decrease to a normal level and, as a result, NGF could rescue the cells. On the other hand, intracellular calcium levels would be further increased by transmembrane calcium influx when NGF was applied to the culture in the presence of extracellular calcium, which might potentiate cell death. This explanation implies that an influx of extracellular calcium, with subsequent cellular calcium overload, would be the cause of the NGF paradox phenomena we observed. To examine whether an influx of extracellular calcium would be the major cause for the cell death potentiated by NGF, we investigated the effect of nifedipine, which is one of the well-known L-type calcium channel blockers (Fig. 3B). Five nM nifedipine completely prevented the NGF-induced cell death. Such a blockade effect of nifedipine also occurred in a concentration-dependent manner, since 5 nM nifedipine prevented cell death significantly ($p<0.05$, $N=4$) more than 1 nM nifedipine did. All these results strongly indicate that a transmembrane calcium influx may be the major cause for the NGF-induced cell death.

Cheng and his colleagues (1993) suggest, as one of the biochemical mechanisms underlying the neuroprotective role of growth factors that they observed, that NGF prevents the late rise in intracellular calcium which is induced by energy deprivation. However, as we pointed out, Rich and Hallowell (1990) suggested that NGF itself should increase intracellular calcium in peripheral neurons by a transmembrane calcium influx and the release of calcium from intracellular stores. It is, therefore, highly probable that in cells exposed to a glucose-deprived condition NGF may cause the increase of intracellular calcium by transmembrane calcium influxes, which results in the potentiation of cell death. However, it is still premature to be more definite

on whether or not the paradoxic NGF phenomenon can be directly mediated by the chanegs in intracellular calcium levels until changes in intracellular calcium levels are directly measured. Experiments are currently under-way to examine the temporal changes of intracellular calcium levels in cells deprived of glucose.

NGF: a rescurer or a killer?

This study shows a differential effect of NGF on cell survivals. PC12 cells treated with NGF before the onset of glucose withdrawl could be partially rescued from hypoglycemic damages by NGF applied under glucose-deprived conditions (Fig. 1). However, NGF potentiated cell death when being applied only after the onset of hypoglycemia (Fig. 2). One of the biochemical processes induced by NGF is to turn on genes for proteins responsible for an intracellular calcium-buffering system. Masiakowski and Shooter (1988) showed in PC12 cells that NGF induces mRNAs coding for peptides with strong sequence homology to a family of calcium-binding proteins. It is, therefore, possible that cells pretreated with NGF have a mature calcium-buffering system and survive more against the sudden increase of intracellular calcium induced by hypoglycemic insults. On the other hand, PC12 cells which have not yet been exposed to NGF may still have an immature calcium-buffering system and survive less against the hypoglycemic insults. Morphological changes and biosynthetic processes led by NGF in PC12 cells are accompanied by an increased energy consumption (Morelli *et al.*, 1986). Thus, the addition of NGF to these cells in energy-deprivation may be another insult in that NGF increases intracellular calcium and energy consumption to mature a calcium-buffering system. The mechanism for the differential actions of NGF is still far from being completely understood in this study. However, we strongly suggest here that NGF as a therapeutic drug against neuronal diseases should be very carefully considered before use.

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