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Cytoprotective Role of Intracelluar Iron on Serum-starved PC12 Cells under NGF

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ABSTRACT

Nerve growth factor (NGF) is known to produce nitric oxide (NO) in PC12 cells, and an iron-NO complex such as dinitro-iron complex (DNIC) is found to keep the cells from apoptosis by inhibiting their caspase activation. Previously we observed that serum-starved PC12 cells maintained their viability only for a couple of days even in the presence of NGF. Therefore, we examined whether any increase of intracellular iron in serum-starved PC12 cells could block the cell death even in the presence of NGF (N-death). Iron successfully prevented the N-death and decreased the amount of NO produced by NGF. Oxy-hemoglobin, an NO scavenger, could also block the N-death. All these results suggest that iron block the N-death by reducing the NO accumulated in a cell under a continual presence of NGF.

Key words: death, Fe, iron-NO complex, NO, NOS

INTRODUCTION

Although NO is well established as a potent apoptosis inducer in several types of cells (Terenzi et al., 1995), NO appears to inhibit apoptosis in some cell systems such as hepatocytes by inhibiting caspase activity through S-nitrosylation of the active site of the enzyme (Kim et al., 1997a, 1997b; Dimmler et al., 1997; Kim et al, 1998). Cellular non-heme iron levels are important in that NO inhibits apoptosis. For example, iron-rich hepatocytes and iron-loaded RAW264.7 cells are more resistant than iron-poor macrophage RAW264.7 cells to S-nitroso-N-acetyl-DL-penicillamine (SNAP)-induced cell death (Kim et al., 1997b). Cellular non-heme iron regulates the level of NO by forming dinitro-iron complex (DNIC) that can transfer NO to a cystein residue (S-nitrosylation) and consequently inhibit caspase. This suggestion is further supported by the fact that NO has high affinity for iron (Watts and Richardson, 2000).

Previously we reported that iron promotes the survival and neurite extension of serum-deprived PC12 cells under NGF by enhancing cell attachment (Hong et al., 2003). The effect of intracellular iron on preventing apoptotic cell death appears to result from both removing NO and converting NO to an S-nitrosylating species (Kim et al., 2000). Along with the enhancement of cell attachment, a down-regulation of NO by intracellular iron seems likely important for blocking the death of serum-starved PC12 cells in the presence of NGF (N-death). We therefore examined whether any increase of intracellular iron could block the N-death

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in connection with NO regulation.

MATERIALS AND METHODS

PC12 (rat adrenal pheochromocytoma ATCC No. 1721) cells were purchased from Korean Cell Line Bank. RPMI 1640 medium (RPMI), horse serum, fetal bovine serum, phosphate buffered saline (PBS) and PSN antibiotic mixtures (penicillin, streptomycin, neomycin included in 0.85% saline) were obtained from GIBCO (Grand Island, USA); DAF-2DA from Calbiochem (La Jolla, California, USA); Culture flasks and dishes from Falcon Lab ware (Oxnard, CA, USA). Other chemicals including Nerve growth factor 7S (NGF) was purchased from Sigma Chemical Co (St. Louis, MO, USA). NGF stock solution (1mg/ml) was prepared in PBS containing 0.1% bovine serum albumin and stored in the -20°C freezer until use.

Cell culture

PC12 cells (passage 15～20) were maintained in RPMI supplemented with 10% horse serum, 5% fetal bovine serum, and 0.01% PSN antibiotic mixtures at 37 (C under a humidified atmosphere of 90% air and 10% CO_2. The medium was changed every 2～3 days. For iron preloading, PC12 cells were supplemented with 100 mM $FeCl_2$ for 24 hours before harvest.

Treatment of reagents

PC12 cells in the 250 ml culture flask were washed free of serum by three cycles of centrifugation/resuspension in RPMI. The cells were resuspended in the serum free RPMI containing 100 ng/mL NGF and appropriate amount of test reagents, and then were plated onto 0.01% poly-L-lysine coated 24-well plates with a cell density of 2.5×10^5 cells per well. For the Fe-chelator complex experiments, iron and chelators were mixed before addition. The molar ratios between iron and diethylenetriamine pentaacetate (DTPA), desferrioxamine (DFO), nitrilotriacetic acid (NTA), and 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron) were 1 : 1, 1 : 1, 1 : 4, and 1 : 4, respectively. The stock solutions of iron chelators were prepared in PBS.

Quantitative measurement of cell survival

The cell viability was measured by an 3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium (MTT) assay method as described by Hansen et al. (1989) with minor modifications. Final 2 mM of 3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetraxolium was added to each 24 well and incubated for two hours at 37°C in dark. The formazan precipitate was formed as the MTT was converted by the mitochodrial dehydrogenases. The formazan precipitate was solubilized with a lysis buffer (20% sodium dodecyl sulfate in 50% N, N-dimethyl formamide; pH adjusted to 4.7) and the absorbance was measured at 570 nm by ELISA reader (Molecular Devices, Menlo Park, USA). In the control experiment, the number of cells and the absorbance showed linear relationship up to 5×10^5 cells, indicating that cell number and mitochondrial activity are correlated. Significant differences between groups of normalized data were determined using analysis of variance (ANOVA) and Student's *t* test as appropriate.

Total cellular iron measurements

Total cellular iron was determined using a colorimetric method after acid-permanganate treatment according to Fish (1988). Total cell lysate was incubated with solution containing iron releasing reagent, 2.25% (w/v) $KMnO_4$ in 0.6N HCl, for 2 hours at 60°C. After centrifugation, the samples were added with iron-chelating reagent containing 5 M ammonium acetate, 6.5 mM ferrozine, 13.1 mM neocuproine, 2 M ascorbic acid, and then their absorbances were measured at 562nm by ELISA reader.

DAF-2DA staining

For diaminofluorescein (DAF) staining, cells were plated in the poly-L-lysine coated cover glass. The cells were stained with 10μM DAF-2DA for 2 h at 37°C. DAF fluorescence was observed using a FITC filter set.

RESULTS AND DISCUSSION

Serum withdrawal made PC12 cells degenerated; 1 day after serum-deprivation, 70% of PC12 cells were degenerated and 2 days after serum-deprivation, less than 10% of PC12 cells main-

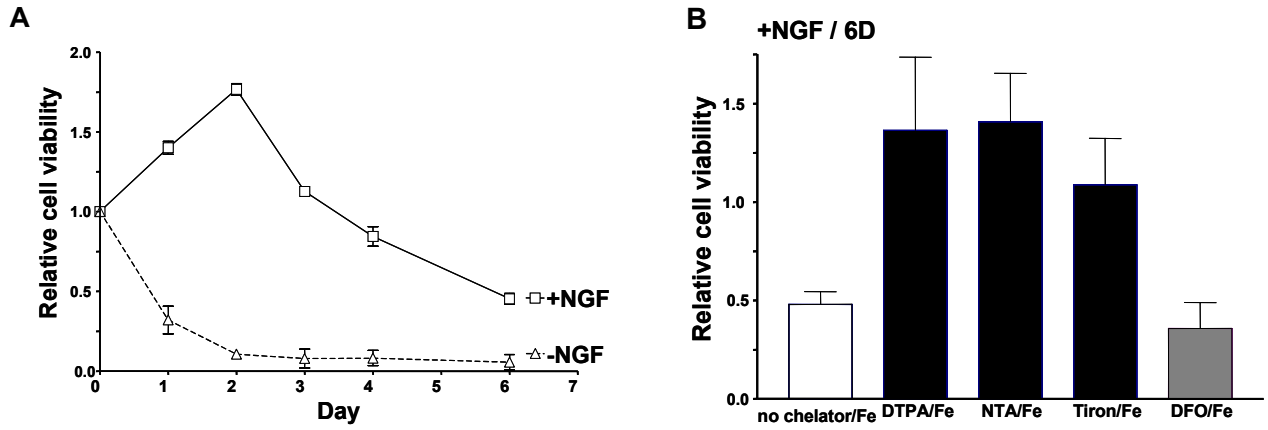

Fig. 1. (A) Effect of NGF on cell survival. Viability of serum-starved PC12 cells was determined by MTT assay either in the absence (△) or the presence (□) of NGF (100 ng/ml). Cell viability at each day was expressed as a relative value compared with the viability at day 0. Normalized data obtained from mean values (n=10); Error bars, SEM. (B) Effect of iron chelator complexes on the viability of the NGF treated PC12 cells. Viability determined 6 days after the addition of NGF (100ng/ml) and iron-chelator complexes. Cell viability was expressed as a relative value compared with the cell viability at day 0. Molar ratios of iron to DTPA or DFO are 1; to NTA or Tiron are 4. The concentration of each chelator used was 100μM. Normalized data obtained from mean values (n=10); Error bars, SEM.

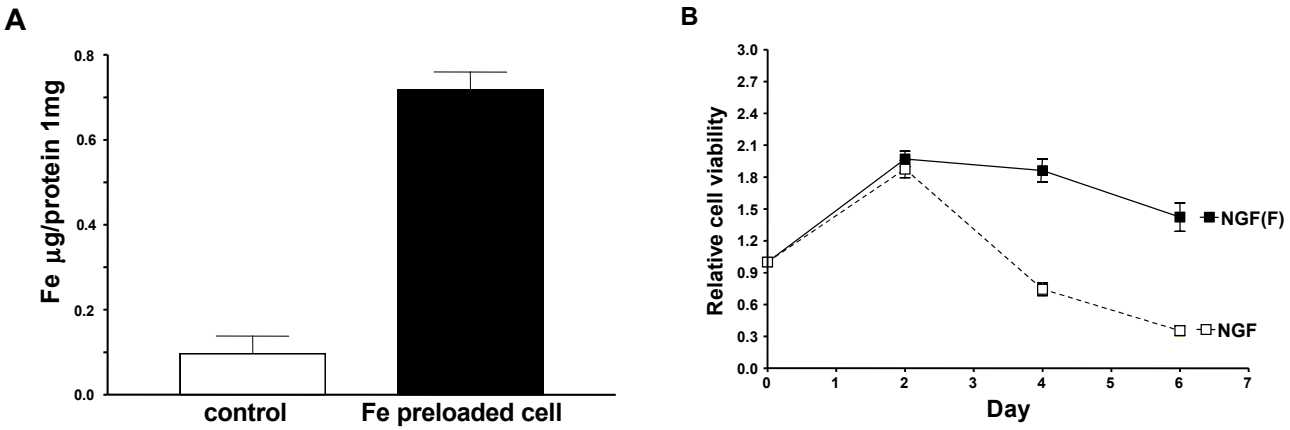

Fig. 2. Effect of iron preloaded into PC12 cells. PC12 cells were incubated with 100μM $FeCl_2$ for 24 hours prior to harvest. (A) shows the concentration of intracellular iron in PC12 cell. (B) shows the viability of iron-preloaded cells as filled squares. Open squares represent the viability of PC12 cells in the NGF supplemented serum free medium. Normalized data obtained form mean values (n=16); Error bars, SEM.

tained their metabolic activities (Fig. 1A, △). NGF could prevent the serum-starved PC12 cells from degenerating (Fig. 1A, □). Viability of the serum-starved PC12 cells appeared to increase in the presence of NGF. However, the increase in cell viability occurred only for 2 days after NGF treatment. After then, the viability of PC12 cells was exponentially declined even in the presence of NGF, supporting the preview study (Greene, 1978) that the duration of PC12 cell culture is dependent on extracellular matrix used.

To test whether intracellular iron might have cytoprotective effect on PC12 cells, the intracellular level of iron was increased by 2 different ways, the use of chelator-iron complex and direct incubation with iron. Iron was known to be taken up into rat hepatocytes when Fe (III)-DTPA was applied (Scheiber and Goldenberg, 1996). Thus, 4 different iron-chelator complexes were used to transport iron into PC12 cells. Three iron-chelator complexes except iron-DFO complex prevented cell death occurring even in the presence of NGF (Fig. 1B). The

iron-DFO showed virtually no effect on cell survival. Since DFO has extremely high affinity for iron (Liu et al, 1999), it seems likely that iron may be hardly released from this complex. In another way, iron was loaded into PC12 cells directly, instead of transporting it into PC12 cells, by incubating them with 100μM $FeCl_2$ for 24 h prior to NGF treatment. Iron preloading significantly increased the intracellular iron content as shown in Fig. 2A; normal PC12 cells contained 0.096μg Fe/protein 1 mg and iron preloaded PC12 cells contained 0.718μg Fe/1 mg protein. As shown in Fig. 2B, pre-incubation of PC12 cells with iron significantly inhibited the N-death and maintained the cell viability for 6 days. These data strongly suggest that the increase of intracellular iron help cell survival in the NGF-treated PC12 cells.

One of the mechanisms suggested for the cytoto-protective role of iron is to form an NO-iron complex that inhibits caspase activity by nitrosylating cystein residues of the enzyme (Kim et al., 2000). Therefore, we examined pharmacologically, using an NO scavenger, whether or not NO involved in the cytoprotective action of iron. When 1 mg/ml oxy-hemoglobin, an NO scavenger, was added to culture medium in the presence of NGF, the viability of the cells became higher than that cultured in the medium without oxy-hemoglobin (Fig. 3A). Such an effect of oxy-hemoglobin was dependent on the concentrations of the scavenger used (Fig. 3B; n=4. $p<0.05$), suggesting that NO apparently involve in the cell death. It is worth noting that the cytoprotective effect of NO scavenger appeared only in the presence of NGF. Accordingly, it is possible that successive accumulation of NO generated by NGF may be responsible for the cell death.

To test whether iron decreased the amount of NO generated by NGF, we examined NO generation in PC12 cells directly by the use of DAF, a novel fluorescent indicator for NO. The fluorescent chemical transformation of DAFs is based on the reactivity of the aromatic vicinal diamines with NO in the presence of dioxygen. The N-nitrosation of DAFs yield highly green-fluorescent triazole forms (Kojima et al. 1998). In the presence of serum, cells were not stained at all (Fig. 4A). On the other hand, all cells strongly stained with green fluorescence through all over the cell body at 2nd (Fig. 4B, D) and 6th day (Fig. 4C, E) after serum deprivation either in the absence of serum or in the presence of NGF. Although it was difficult to measure the NO content quantitatively, the fluorescent intensity of PC12 cells preloaded with iron looked likely lesser than that of PC12 cells without iron (Fig. 4F, G). Therefore, all these results suggest that iron might decrease the intracellular level of NO generated by NGF.

In summary, iron preloading significantly inhibits

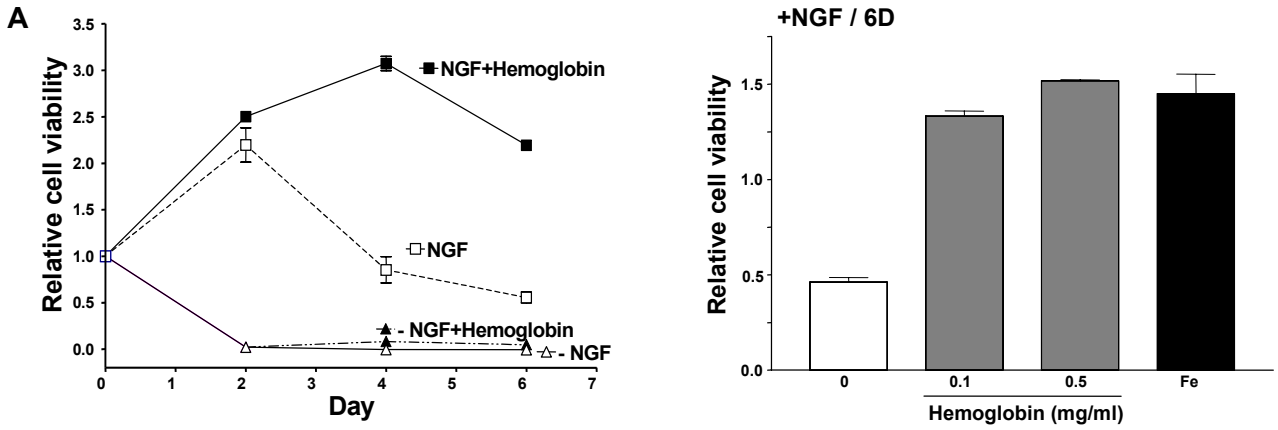

Fig. 3. (A) Effect of oxy-hemoglobin on the viability of NGF treated PC12 cells. One mg/ml oxy-hemoglobin prevented the cell death occurring even in the presence of NGF. Relative cell viability of PC12 cells in serum free medium, serum free medium with oxy-hemoglobin, NGF, and NGF with oxy-hemoglobin are denoted in △, ▲, □, ■, respectively. Normalized data obtained from mean values (n=4); Error bars, SEM. (B) Concentration dependency of hemoglobin action. Two days after incubation, hemoglobin was added to the media. Cell viability was determined at sixth day after addition of NGF on the serum-deprived PC12 cells. Normalized data obtained from mean values (n=4); Error bars, SEM.

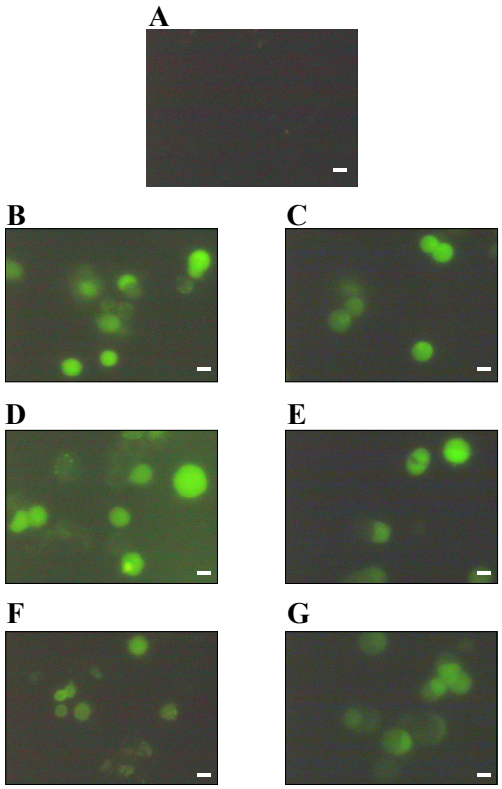

Fig. 4. NO production in PC12 cells. NO generation was examined using DAF (10μm). (A) PC12 cells in the presence of serum, (B) PC12 cells observed at 2 days after serum-deprivation, (C) PC12 cells observed at 6 days after serum-deprivation, (D) serum-deprived PC12 cells at 2 days after NGF addition, (E), serum-deprived PC12 cells at 6 days after NGF addition, (F) serum-deprived PC12 cells at 2 days after NGF and iron addition, (G) serum-deprived PC12 cells at 6 days after NGF and iron addition. Scale bar, 20μM. Magnification ratio ×100.

the PC12 cell death in the NGF supplemented serum free medium, possibly by decreasing the NO induced by NGF.

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