Correlation between Neuritogenic Action of Nitric Oxide and the Rate of Nitric Oxide Production in PC12h Cells

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To investigate the effects of the nitric oxide (NO) generating rate on neuritogenesis, we compared the neuritogenic activity of several NO donors with varying generating rates; NOR1 (t1/2 = 1.8 min), NOR2 (t1/2 = 28 min), NOR3 (t1/2 = 30 min), and NOR4 (t1/2 = 60 min). Each NO donor promoted neurite outgrowth in PC12h cells in a concentration-dependent manner. However, the profiles were not dependent on the NO-generating rate. The neuritogenesis promoted by NOR4 was inhibited by the NO trapping agent carboxy-2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, sodium salt (PTIO). NOR2 and NOR3, which have similar generating rates, were more toxic than NOR1 and NOR4 in PC12h cells. NOR2 and NOR3 induced cytotoxicity at concentrations of 100 μM and above, while NOR1 and NOR4 induced cytotoxicity at concentrations of 200 μM and above. Preconditioning medium containing these NO donors in the absence of cells were less cytotoxic towards PC12h cells. Therefore, we conclude that the neuritogenic action of NO in PC12h cells is dependent on a suitable generating rate, but not dependent on the amount of NO.

Key words —— PC12h cell, neuritogenesis, neuritotoxicity

INTRODUCTION

Rat pheochromocytoma PC12 cell lines are well-characterized NGF-responsive cell lines and differentiate to neuronal cells that activate the ras-extracellular signal-regulated kinase (ERK) pathway through nerve growth factor (NGF) receptor, trkA.[1)] Recently, it has been demonstrated that NGF enhances nitric oxide (NO) production by the induction of NO synthase (NOS) in PC12 cells[2)] and suggested that the NO participates in the neuronal differentiation of PC12 cells mediated by NGF. In PC12 cells, it has been further shown that NO donors also enhanced the neuritogenic effects of NGF, however, NO donors did not induce PC12 cell neurites in the absence of NGF.[3,4)] Meanwhile, we and other investigators have previously reported that several NO donors induced neurites in PC12h cells[5)] and PC12W,[6)] a subclone of PC12 cells, and in NG108-15.[7)]

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NO plays an important role in intracellular and intercellular signaling events such as neural development, synaptic plasticity, and neuroendocrine secretion by low level NO production through neuronal and/or endothelial NOS activation.[8)] Inducible NOS produces high levels of NO and has been implicated in neuronal cell death. Therefore, the concentration of NO around the cells seems to be very important for the physiological function of neuronal cells.

In the present report, we have investigated the correlation between NO generating rate and neuritogenic activity using several NO donors that have various NO generating rates in PC12h cells.

MATERIALS AND METHODS

Materials —— PC12h cells were a kind gift from the late Dr. H. Hatanaka. The NO donors NOR1–NOR4 and the NO scavenger carboxy-2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, sodium salt (PTIO) were purchased from Dojindo, Co. Ltd. (Kumamoto, Japan). The NO-sensitive fluorescent dye diaminofluorescein-2

diacetate (DAF-2DA) was purchased from Daiichi Pure Chemicals, Co. Ltd. (Tokyo, Japan).

Measurement of NO Production —— NO was measured using diaminofluorescein-2 (DAF-2), an NO-sensitive fluorescent probe.[9] Time-dependent NO production was monitored at 37°C at excitation and emission wavelengths of 495 and 515 nm, respectively.

Cell Culture —— PC12h cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5%(v/v) horse serum and 5%(v/v) precolostrum calf serum as previously reported.[5] Briefly, PC12h cells were plated in 35 mm culture dishes coated with collagen at a density of 4 $\times 10^4$ cells in 2 ml of medium per dish. After 24 hr of culture the growth medium was replaced by serum-free DMEM/Ham's F12 (1 : 1) medium supplemented with sodium selenate, transferrin, insulin, progesterone, and vehicle [dimethyl sulfoxide (DMSO), unless otherwise noted] in the case of the control or one of the treatment compounds. Preconditioning medium treated with NORs to evaluate the toxic effect was prepared by incubation with an NOR (100 μM) for 24 hr at 37°C.

Evaluation of Neurite Outgrowth —— Neurite outgrowth of PC12h cells was evaluated after treatment for 24 hr, unless otherwise specified, by measuring the longest neurite of individual cells as previously reported.[5] The average neurite length of 100 cells in each treatment was calculated. Statistical analyses were performed using analysis of variance (ANOVA) and Student's *t*-test.

RESULTS AND DISCUSSION

Treatments with 4 NO donors, NOR1, NOR2, NOR3, and NOR4, for 24 hr induced neurite outgrowth in PC12h cells in a concentration-dependent manner (Fig. 1). This neuritogenic activity and NO production of NOR4 were greatly inhibited by the NO-trapper carboxy-PTIO, as shown in Fig. 2. These results indicated that exogenous NO from the donors has neuritogenic activity in PC12h cells. Furthermore, this neurite outgrowth was observed even for very short treatment times (one hour after) (Fig. 3). These results suggest that the trigger for neuritogenesis induced by NO begins at the initial phase of NO generation in PC12h cells. Therefore, it is expected that the neurite outgrowth depends on the concentration of the initially-generated NO, which is dependent on the half-life of the NO donor.

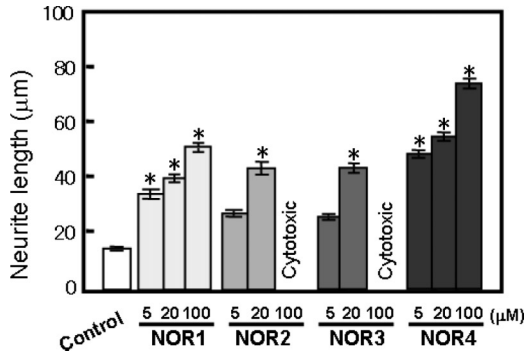

Fig. 1. NORs — Induced Neurite Outgrowth

PC12h cells were treated with a NOR (0–100 μM) or its vehicle (DMSO: control) for 24 hr. Details of the method are described in the MATERIALS AND METHODS. Data are presented as the mean ± SEM. *$p < 0.01$ *versus* control value.

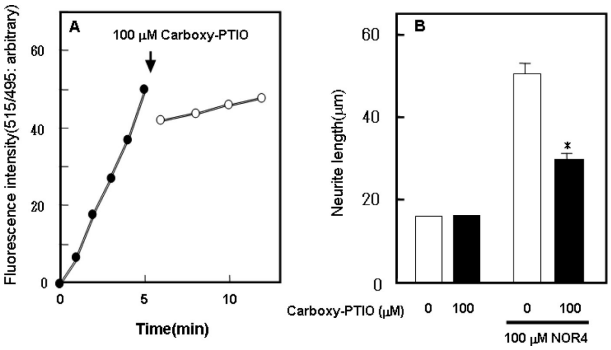

Fig. 2. Effects of NO-Trapper on NO Generation (A) and Neuritogenic Activity (B) of NOR4

(A) Time course of NO-generation was monitored by 10 μM DAF-2 in neutral buffer (Krebs-Ringer phosphate, pH 7.2). The reaction was started by the addition of 25 μM NOR4 and was followed 5 min later by the addition of 100 μM carboxy-PTIO. (B) PC12h cells were pretreated with 100 μM carboxy-PTIO and then 30 min later the cells were treated with 100 μM NOR4 for 24 hr. Data are presented as the mean ± SEM. *$p < 0.01$ *versus* NOR4 alone. Details of the method are described in the MATERIALS AND METHODS.

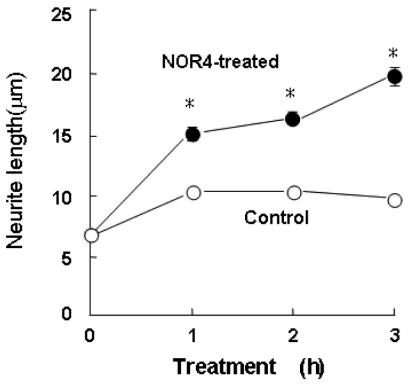

Fig. 3. Neurite Outgrowth in the Short-Term

PC12h cells were treated with 100 μM NOR4. Details of the method are described in the MATERIALS AND METHODS. Data are presented as the mean ± SEM. *$p < 0.01$ *versus* each respective control value.

As shown in Fig. 4, the half lives of the NO donors used in these experiments generated NO were as follows, NOR1 > NOR2–3 > NOR4. However, when the neuritogenic activities at donor concentrations of 20 μM were compared, the order was NOR4 > NOR2–NOR3 > NOR1. In other words, we were unable to observe a correlation between neuritogenic activity and the amount of NO generated.

NOR2 and NOR3, which had similar generating rates, were more toxic towards PC12h cells than NOR1 and NOR4. NOR2 and NOR3 induced cytotoxicity in PC12h cells at 100 μM or more, while NOR1 and NOR4 showed cytotoxicity at 200 μM or more (Fig. 5). Preconditioning media incubated for 24 hr with these NO donors in the absence of cells did not exhibit any biological effects on PC12h cells. No preconditioning media had either neuritogenic (Fig. 5A) or cytotoxic effects (Fig. 5B), although NOR2 and NOR3 showed cytotoxicity at the same concentration. These results indicate that the degradation products of these NO donors were not due to the induction of cytotoxicity and suggest that the degree of NO-induced neuronal damage is not correlated with either the amount of NO generated or the rate of generation. Though further analysis is necessary, the reason for this may be the difference in the amount of $ONOO^-$ generation since it

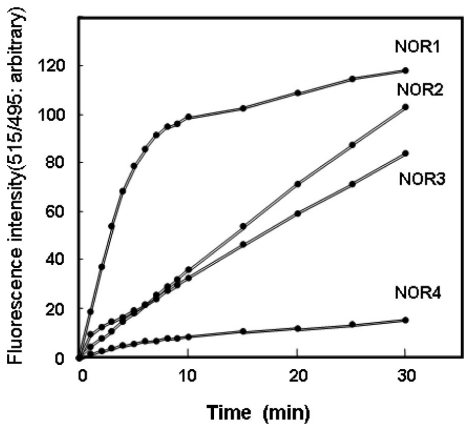

Fig. 4. NO-Generation Rates for NOR1–NOR4

Time courses of NO-generation were monitored by 10 μM DAF-2 in Krebs-Ringer phosphate buffer, pH 7.2. The reaction was started by the addition of 25 μM NORs. Details of the method are described in the MATERIALS AND METHODS.

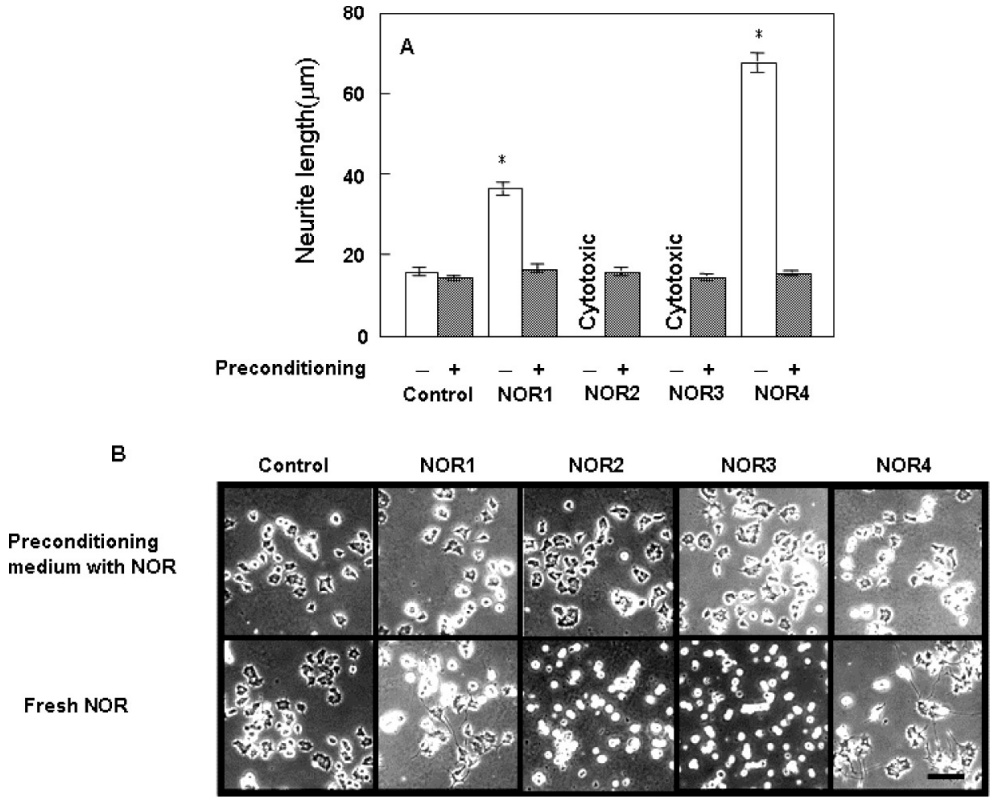

Fig. 5. Evaluation of Biological Activity of the Preconditioning Medium on PC12h Cells

PC12h cells were treated with 100 μM of an NOR and the corresponding preconditioning medium. Neuritogenic activity (A) and morphology (B) were analyzed after 24 hr. Data for (A) are the mean ± SEM. *$p < 0.01$ *versus* control (vehicle). Scale bar in (B) = 100 μm. Details of the method are described in the MATERIALS AND METHODS.

has been previously reported that the toxicity is not due to NO but rather to $ONOO^-$.[10] In other words, it would appear that the rate of NO generation may be sufficient to result in some $ONOO^-$ production.

In conclusion, we have investigated the correlation between the NO generating rate and neuritogenic activity with several NO donors which have various NO generating rates in PC12h cells, and shown that both the neuritogenic and neurotoxic effects were not simply correlated to the amount and rate of NO production.

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