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Fundamental role of nitric oxide in neuritogenesis of PC12h cells

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1 We investigated the neuritogenic action of nitric oxide (NO)-generating agents and their mechanisms of action in a subclone of rat pheochromocytoma, PC12h cells.

2 NO donors such as sodium nitroprusside (SNP, 0.05–1 μM), NOR1 (5–100 μM), NOR2 (5–20 μM), NOR3 (5–20 μM), NOR4 (5–100 μM), or *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP, 10–100 μM) significantly induced neurite outgrowth.

3 NOR4-induced neurite outgrowth was accompanied by expression of neurofilament 200 kDa subunit (NF200) protein, an axonal marker, and was significantly inhibited by an NO scavenger, a soluble GC inhibitor, and a PKG inhibitor: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (carboxy-PTIO, 20–100 μM), 1*H*-[1,2,4]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ, 100 μM) and KT5823 (0.2–1 μM), respectively.

4 The intracellular cGMP concentration of cells was markedly increased by treatment with NOR4 (100 μM).

5 A mitogen-activated protein kinase (MAPK) kinase inhibitor, PD98059 (10–50 μM), abolished the NOR4-induced neurite outgrowth. In agreement with this observation, NOR4 did phosphorylate extracellular signal-regulated kinase (ERK) 1 and 2, substrates of MAPK kinase.

6 A membrane-permeable cGMP analog, 8-Br-cGMP (1 mM) also induced significant neurite outgrowth. The 8-Br-cGMP-induced neurite outgrowth was almost completely inhibited by both KT5823 (0.5 μM) and PD98059 (50 μM). Moreover, sustained ERK phosphorylation was observed in the 8-Br-cGMP-treated PC12h cells.

7 These results suggest that NO itself has the ability to induce neurite outgrowth and that NO-induced ERK activation involves the NO-cGMP-PKG signaling pathway in PC12h cells.
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Abbreviations: BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide; DMEM, Dulbecco's modified Eagle's medium; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; IBMX, 3-isobutyl-1-methylxanthine; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide; NF200, neurofilament 200 kDa subunit; NGF, nerve growth factor; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; sGC, soluble guanylyl cyclase; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; SNP, sodium nitroprusside

Introduction

In mammalian cells, nitric oxide (NO) is synthesized from L-arginine by NO synthase (NOS) of which there are three isoforms; neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Moncada & Higgs, 1993). NO has been implicated in a variety of biological actions such as vasodilation, inhibition of platelet aggregation, neurotransmission, and cell survival (Ohkuma & Katsura, 2001; Thippeswarny *et al*., 2001; Klinge *et al*., 2003; Xu *et al*., 2003). Nevertheless, it has also been reported that NO is a toxic factor mediating cell death in a number of cell types (Golde *et al*., 2002; Klein & Brüne, 2002; Yamaoka *et al*., 2002). In the former case, NO seems to exert this action by an increase in the cGMP concentration *via* activation of soluble guanylyl cyclase (sGC). In the latter case, it seems that NO reacts with super oxide anion to give cytotoxic peroxynitrite.

The rat pheochromocytoma PC12 cell line has been used extensively as a model for studying neuronal differentiation (Greene & Tischler, 1976). PC12 cells respond to neurotrophic factors, such as nerve growth factor (NGF) and fibroblast growth factor (FGF), by differentiating into sympathetic neuron-like phenotypes characterized by neurite outgrowth and expression of many neuron-specific proteins (Greene & Tischler, 1976; Burstein *et al*., 1982; Cowley *et al*., 1994). The mechanisms involved in NGF-induced differentiation of PC12

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cells have been explored in detail. It has been well established that NGF activates its high-affinity receptor, TrkA, and then activates the Ras-extracellular signal-regulated kinase (ERK) pathway (Marshall, 1995).

It has been demonstrated that NGF induces NO production by the induction of all three NOS isoforms in PC12 cells, suggesting that NO participates in neuronal differentiation of these cells by NGF (Peunova & Enikolopov, 1995). In PC12 cells, it has been further shown that an NO donor enhances the neuritogenic effect of NGF; however, it has also been reported that the NO donor itself does not display the effect in the absence of NGF (Hindley *et al*., 1997). On the contrary, in a preliminary investigation, we observed that several NO donors themselves induce neurite outgrowth in PC12h cells. PC12h is a subclone of PC12 and has neuritogenic activity equal to that of parental PC12 and NGF-responsible tyrosine hydroxylase activity (Hatanaka, 1981).

We have recently reported that NGF induces neurite outgrowth *via* NO-cGMP signaling pathway in PC12h cells (Yamazaki *et al*., 2001). In addition, stimulation of the NO-cGMP signaling pathway consequently induces activation of ERKs. These results suggest that NO plays an important role in inducing differentiation of PC12h cells. Therefore, in this study, we have investigated the direct effect of NO on neuritogenesis using several NO donors and analyzed its mechanisms using specific inhibitors of the NO pathway. Our results indicate that exogenous NO itself can induce neurite outgrowth by ERK activation through NO-cGMP-PKG pathway in PC12h cells.

Methods

Cell culture and drug treatment

PC12h cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v v^{-1}) horse serum and 5% (v v^{-1}) precolostrum calf serum containing 0.1 mg ml^{-1} kanamycin in a humidified atmosphere of 90% air–10% CO_2 at 37°C. The cells were plated at a density of 1×10^4 cells cm^{-2} on 35 mm-diameter dishes coated with collagen type I. After 24 h of culture, the medium was replaced by serum-free DMEM/Ham's F12 medium supplemented with 30 nM sodium selenate, 5 μg ml^{-1} transferrin, 5 μg ml^{-1} insulin and 20 nM progesterone containing vehicle for control or appropriate compounds as specified in the results for treatment.

Evaluation of neurite outgrowth

Neurite outgrowth of PC12h cells was evaluated as previously reported (Yamazaki *et al*., 2001). Briefly, after treatment with drugs for 24 or 48 h, we measured the length of the longest neurite in individual cells of 100 cells in 10 random fields from two culture dishes. Each neurite was measured with an image processor system (model XL-500, Olympus, Tokyo, Japan) attached to a phase-contrast microscope (Olympus) using an application software (Image Command 4198, RATOC Engineering, Tokyo, Japan). The mean values of neurite length were normalized relative to that of the cells treated with vehicle alone. The results were expressed as the mean ± s.e. of three separate experiments.

Measurement of intracellular cGMP

PC12h cells (10^6 cells) were pretreated with 100 μM 3-isobutyl-1-methylxanthine (IBMX), a nonselective inhibitor of PDEs, for 30 min and then 100 μM NOR4 was added for 10–30 min. Intracellular cGMP concentration was determined with a cGMP enzyme-immunoassay kit after cells were lysed with 0.5% (w v^{-1}) dodecyltrimethylammonium bromide according to the manufacturer's protocol. The protein content of the samples was determined according to the method of Lowry *et al*. (1951) using BSA as a standard.

Western blot analysis

Western blot analysis was carried out as previously described (Yamazaki *et al*., 2001). Briefly, PC12h cells treated with several conditions as mentioned in the Results and were rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed with lysis buffer, 50 mM Tris/HCl (pH 7.4), 0.5 mM EGTA, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride, 1 mM dithiothreitol, phosphatase inhibitor cocktail I and II, protease inhibitor cocktail, and 0.1% (v v^{-1}) Nonidet P-40. The cell lysates obtained (100 μg for neurofilament protein and 10 μg for ERK) were separated on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane. The membrane was hybridized with anti-neurofilament 200 kDa subunit (NF200) monoclonal antibody (clone NE14), anti-Na^+, K^+-ATPase α1 subunit monoclonal antibody (clone C464.6), anti-mitogen-activated protein kinase (MAPK) (ERK1 and 2) polyclonal antiserum, or anti-active MAPK polyclonal antiserum. Anti-Na^+, K^+-ATPase α1 subunit monoclonal antibody was used to assess the quality of protein loading. Alkaline phosphatase-conjugated anti-mouse Ig G antiserum or alkaline phosphatase-conjugated anti-rabbit Ig G antiserum was used as the secondary antibody. The immunoreactive signals were detected using a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) Phosphatase Substrate System detection kit. The developed membranes were digitized and composed with Adobe Photoshop (Adobe Systems, Mountain View, CA, U.S.A.). The blots shown are representative of 3–5 independent experiments.

Materials

DMEM was purchased from Nissui Pharmaceutical (Tokyo, Japan). Horse serum and Ham's F12 were purchased from Gibco BRL (Rockville, MD, U.S.A.). Precolostrum calf serum was purchased from Mitsubishi Kasei (Tokyo, Japan). Kanamycin was purchased from Meiji Seika Kaisha (Tokyo, Japan). Collagen type I was purchased from Elastin Products (Owensville, MO, U.S.A.). KT5823 and PD98059 were purchased from Nacalai Tesque (Kyoto, Japan) and Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.), respectively. NOR1-4, *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), SIN-1 and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (carboxy-PTIO) were obtained from Dojin Chemical (Kumamoto, Japan). Transferrin, progesterone, sodium nitroprusside (SNP), 1*H*-[1,2,4]oxadiazolo [4,3-*a*]quinoxalin-1-one (ODQ) and IBMX were purchased from Wako Pure Chemical (Osaka, Japan). Insulin, NGF(7S),

8-Br-cGMP, phosphatase inhibitor cocktail I and II, protease inhibitor cocktail (general use), anti-NF200 monoclonal antibody, anti-MAPK (ERK1 and 2) polyclonal antiserum, alkaline phosphatase-conjugated anti-mouse Ig G antiserum, and alkaline phosphatase-conjugated anti-rabbit Ig G antiserum were obtained from Sigma Chemical (St Louis, MO, U.S.A.). Anti-active MAPK polyclonal antiserum and anti-Na^+, K^+-ATPase α1 subunit monoclonal antibody were obtained from Promega (Madison, WI, U.S.A.) and Upstate Biotechnology (Lake Placid, NY, U.S.A.), respectively. Immobilon-P PVDF membrane was purchased from Millipore (Bedford, MA, U.S.A.). cGMP enzyme-immunoassay kit was purchased from Amersham Pharmacia Biotech (Tokyo, Japan). BCIP/NBT Phosphatase Substrate System detection kit was obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MO, U.S.A.). All other chemicals were of the highest grade of purity.

Statistical evaluation

The results of neurite outgrowth and intracellular cGMP concentration are expressed as the mean ± s.e. for three and four independent experiments, respectively. One-way ANOVA and Scheffe's test as *post hoc* tests were used to test for differences between experimental groups. When the *P*-value was <0.05, the difference was considered to be significant.

Results

Neuritogenesis induced by NO

We examined whether NO itself could induce neurite outgrowth in PC12h cells. NGF, a positive control, induced significant neurite outgrowth accompanied by formation of networks at concentrations of 1 and 100 ng ml^{-1} for 24 h in PC12h cells (Table 1). Treatment with NO donors, SNP (Kowaluk *et al*., 1992) and SNAP (Garg & Hassid, 1989), for 24 h caused significant neurite outgrowth in a concentration-dependent manner (Table 1). Four other NO donors with different half-life, NOR1 ($t_{1/2}=1.8$ min), NOR2 ($t_{1/2}=28$ min), NOR3 ($t_{1/2}=30$ min), and NOR4 ($t_{1/2}=60$ min) (Kita *et al*., 1994; 1995; Kato *et al*., 1996), also showed significant neuritogenic activity in these cells (Table 1). When these cells were treated with all NO donors tested here at indicated concentrations for up to 48 h, we could not observe cytotoxicity from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolim bromide (MTT) reduction assay and trypan blue exclusion assay (data not shown). High concentrations of SNP (5 μM and more), SNAP (250 μM and more), NOR1 (200 μM and more), NOR2 (100 μM and more), NOR3 (100 μM and more), and NOR4 (200 μM and more) were found to be cytotoxic in these cells. It is known that NO physiologically reacts with superoxide anion to give peroxynitrite and that peroxynitrite is associated with toxicity. To confirm whether the NO donor-induced neurite outgrowth results from NO itself or peroxynitrite produced from NO, we examined the neuritogenic activity of the peroxynitrite generator, SIN-1 (Saran *et al*., 1990). SIN-1 did not exhibit any activity at a concentration range of 5–250 μM (Table 1) and made the cells shrink and float at concentrations higher than 250 μM. Moreover, no effect of the degradation products after NO release from NOR1–4 on neuritogenesis were observed by treatment of the cells with medium preincubated with 100 μM NOR1–4 in the absence of cells for 24 h at 37°C (data not shown). These results suggest that the neurite outgrowth induced by NO donors is due to NO itself, and not peroxynitrite.

Table 1 NO donors induced neurite outgrowth in PC12h cells

Compounds	*Concentrations*	*Relative neurite length*
	0 (ng ml^{-1})	1.00 ± 0.03 (26.6 ± 0.9 μm)
NGF	1	3.28 ± 0.05**
	100	3.86 ± 0.06**
	0 (μM)	1.00 ± 0.02 (26.3 ± 0.6 μm)
SNP	0.05	1.50 ± 0.10*
	0.1	1.81 ± 0.08**
	1	1.80 ± 0.05**
	0 (μM)	1.00 ± 0.07 (17.4 ± 1.3 μm)
SNAP	10	1.94 ± 0.22**
	50	2.80 ± 0.22**
	100	3.53 ± 0.20**
	0 (μM)	1.00 ± 0.03 (15.3 ± 0.4 μm)
NOR1	5	2.16 ± 0.08**
	20	2.44 ± 0.14**
	100	3.27 ± 0.09**
NOR2	5	1.70 ± 0.29
	20	2.80 ± 0.16**
NOR3	5	1.65 ± 0.15
	20	2.80 ± 0.20**
NOR4	5	3.09 ± 0.03**
	20	3.59 ± 0.13**
	100	4.61 ± 0.14**
SIN-1	0 (μM)	1.00 ± 0.11 (28.0 ± 3.0 μm)
	5	0.98 ± 0.05
	20	0.96 ± 0.04
	100	0.88 ± 0.04
	250	1.12 ± 0.03

PC12h cells were treated with the indicated concentrations of NGF, NO donors, peroxynitrite generator, or their vehicle (0) for 24 h. These compounds and their vehicle were applied once for the treatment. Relative neurite length was then evaluated as described in the Methods. NGF served as a positive control. NGF was dissolved in PBS containing 0.1% (w v^{-1}) bovine serum albumin, SNP was dissolved in distilled water, and SNAP and NORs were dissolved in dimethylsulfoxide. SIN-1 was dissolved in 0.6 M hydrochloride. The final concentrations ($<0.2\%$ (v v^{-1})) of these solvents showed no cytotoxicity and neuritogenesis. The data shown represent the mean ± s.e. from three independent experiments. The values in the parentheses are the mean values of neurite length. *$P<0.05$, **$P<0.01$ *versus* corresponding values of vehicle alone (ANOVA, Scheffe's test).

Induction of neurofilament protein by NO treatment

To analyze the mechanism of the neurite outgrowth induced by NO, we used NOR4 which had the most effective neuritogenic action of all NO donors tested. We applied NOR4 at a concentration of 100 μM. The NOR4-induced neurites were relatively straight and frequently had growth

cone-like constructions and formed networks (Figure 1a). One of the markers for neuronal differentiation is the expression of neurofilament isoforms (Lindenbaum *et al*., 1987; Gold *et al*., 1991; Lee & Cleveland, 1996). We observed that the expression of NF200, an axonal marker, was drastically increased after treatment with NOR4 for 24 h in PC12h cells (Figure 1b).

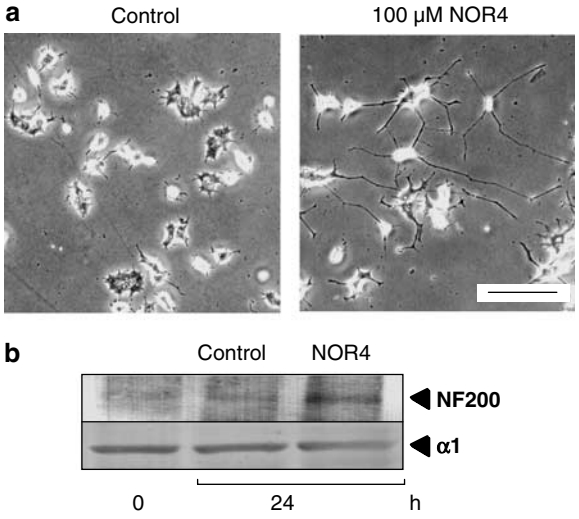

Figure 1 NOR4-induced neuronal differentiation in PC12h cells. PC12h cells were treated with 100 μM NOR4 or its vehicle (Control) for 24 h. (a) Microphotographs were taken. Scale bar indicates 100 μm and is applied to both microphotographs. (b) The prepared cell extracts (100 μg protein lane^{-1}) were subjected to 6% SDS–PAGE and immunoblotted with antibodies against NF200 and Na^+, K^+-ATPase α1 subunit (α1). α1 is used to assess the quality of protein loading. The blots at 0 h were from untreated cells. The data shown are typical of four independent experiments that indicated similar results.

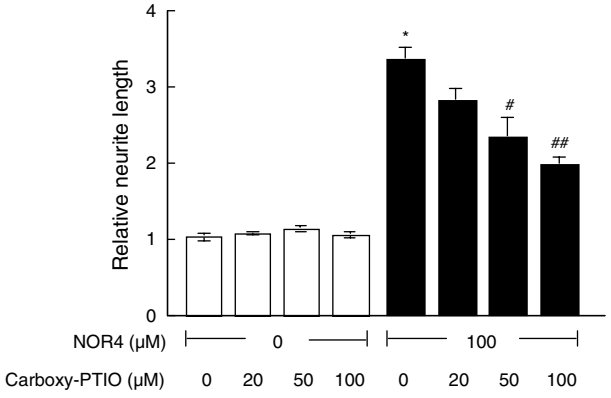

Figure 2 NO scavenger inhibited NOR4-induced neurite outgrowth in PC12h cells. PC12h cells were treated with the indicated concentrations of carboxy-PTIO or its vehicle (0) plus 100 μM NOR4 or its vehicle (0) for 24 h. Relative neurite length was then evaluated as described in Methods. Carboxy-PTIO and NOR4 were dissolved in PBS and dimethylsulfoxide, respectively. The final concentrations ($<0.1\%$ (v v^{-1})) of these solvents showed no cytotoxicity and neuritogenesis. The data shown represent the mean$\pm$s.e. of three independent experiments. The mean value of neurite length was 15.4 ± 0.9 μm in vehicle-treated cells. $^*P<0.01$ *versus* vehicle alone and $^{\#}P<0.05$, $^{\#\#}P<0.01$ *versus* NOR4 alone (ANOVA, Scheffe's test).

These findings were similar to those of PC12h cells differentiated by NGF (Yamazaki *et al*., 2004). Thus, it seems that the NOR4-induced neurite outgrowth reflects the neuronal differentiation in PC12h cells.

Inhibition of NO-induced neuritogenesis by NO scavenger

An NO scavenger, carboxy-PTIO (20–100 μM) (Akaike *et al*., 1993), significantly attenuated NOR4-induced neurite outgrowth even when the scavenger was simultaneously applied with NOR4 (Figure 2). However, because of the cytotoxicity of carboxy-PTIO, we failed to observe complete inhibition by this scavenger at concentrations higher than 100 μM. Thus, exogenous NO in itself has neuritogenic activity in PC12h cells.

Involvement of cGMP in NO-induced neuritogenesis

It is well known that NO directly activates sGC and increases intracellular cGMP levels (Schulz *et al*., 1989). The involvement of sGC in neurite outgrowth induced by NOR4 was examined using ODQ, a selective inhibitor of NO-sensitive sGC (Garthwaite *et al*., 1995). ODQ (100 μM) completely blocked the neurite outgrowth induced by NOR4 (5 and 100 μM) (Figure 3a). This suggests that NOR4-induced neurite outgrowth is completely dependent on the activation of sGC. In fact, the NOR4-treated cells had a markedly increased intracellular cGMP concentration compared with untreated cells (Figure 3b).

Inhibition of NO-induced neuritogenesis by PKG inhibitor

It is known that cGMP regulates several effector targets including cation channels, PDEs, and PKG (Schmidt *et al*., 1993). Next, we examined the effect of a PKG inhibitor on NOR4-induced neuritogenesis. KT5823 (0.2–1 μM), an inhibitor of PKG, significantly decreased the neuritogenesis induced by NOR4 in a concentration-dependent manner (Figure 4). On the other hand, KT5823 had no effect on neuritogenesis induced by dibutyryl-cAMP, a membrane-permeable cAMP analog, even at the high concentration of 5 μM (Yamazaki *et al*., 2004), indicating that KT5823 specifically inhibits PKG.

Involvement of ERK activation in NO-induced neuritogenesis

It has been well established that the sustained activation of ERK by NGF is involved in neuronal differentiation of PC12 cells (Fukuda *et al*., 1995; Marshall, 1995). We have recently reported that the sustained activation of ERK is important to neurite outgrowth induced by NGF and that the activation occurs through activation of nNOS and iNOS in PC12h cells (Yamazaki *et al*., 2001). In order to determine the role of ERK in neurite outgrowth induced by NOR4, we treated PC12h cells with PD98059 (Dudley *et al*., 1995), a selective inhibitor of MAPK/ERK kinase (MEK), for 1 h before NOR4 was added. As shown in Figure 5a, inhibition of ERK by PD98059 (10–50 μM) effectively inhibited the NOR4-induced neurite outgrowth in a concentration-dependent manner. In support of this finding, treatment of the cells with NOR4 resulted in greater ERK phosphorylation for at least 60 min than that of untreated cells (Figure 5b). Therefore, it seems that NOR4 as

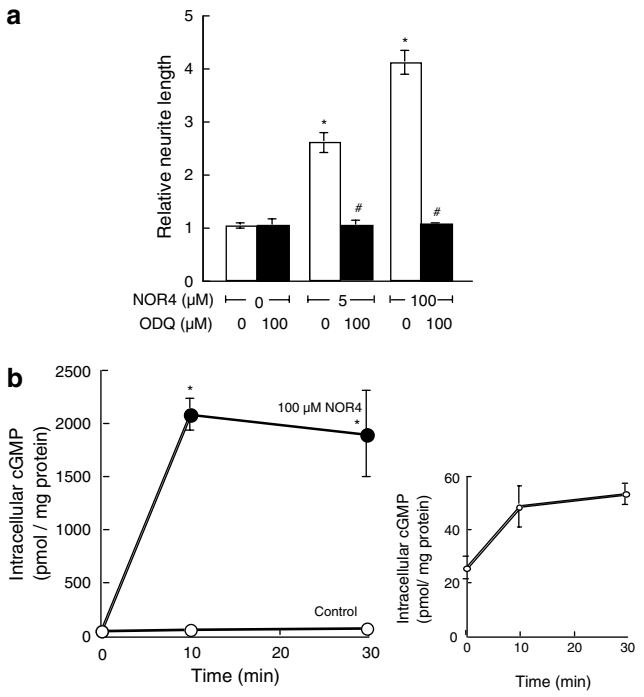

Figure 3 NOR4-induced neurite outgrowth through cGMP production in PC12h cells. (a) PC12h cells were pretreated with 100 μM ODQ or its vehicle (0) for 60 min. The indicated concentrations of NOR4 or its vehicle (0) were then added to the culture. After 24 h, relative neurite length was evaluated as described in Methods. Both ODQ and NOR4 were dissolved in dimethylsulfoxide. The final concentrations ($<0.2\%$ (v v^{-1})) of this solvent showed no cytotoxicity and neuritogenesis. The data shown represent the mean $\pm$ s.e. of three independent experiments. The mean value of neurite length was 18.7 ± 1.0 μm in vehicle-treated cells. $^{*}P<0.01$ *versus* vehicle alone and $^{\#}P<0.01$ *versus* corresponding values of NOR4 alone (ANOVA, Scheffe's test). (b) PC12h cells were pretreated with 100 μM IBMX for 30 min. Then, 100 μM NOR4 or its vehicle (Control) was added to the culture for the indicated periods of time. Intracellular cGMP content was determined with a cGMP enzyme immunoassay kit. The smaller graph is a magnification of the result for the control. The data shown represent the mean $\pm$ s.e. of four independent experiments. $^{*}P<0.01$ *versus* value of time 0 (ANOVA, Scheffe's test).

well as NGF requires activation of ERK to exert its neuritogenic activity in PC12h cells.

Neuritogenesis induced by cGMP

Finally, we examined whether exogenous cGMP can mimic the signaling pathway triggered by NOR4. Treatment of PC12h cells with 8-Br-cGMP, a membrane-permeable cGMP analog, for 48 h induced significant neurite outgrowth in a concentration-dependent manner (0.1–1 mM) (0 mM; 1 ± 0.06, 0.1 mM; 2.01 ± 0.12, 0.5 mM; 2.56 ± 0.13, 1 mM; 2.94 ± 0.14 in relative neurite length, $n = 3$). The neuritogenic activity of 8-Br-cGMP (1 mM) was almost completely blocked in the presence of KT5823 (0.5 μM) or PD98059 (50 μM) (Figure 6a). Moreover, the 8-Br-cGMP-treated cells exhibited progressively enhanced ERK phosphorylation for at least 60 min in contrast to untreated cells (Figure 6b). These findings demonstrate that cGMP activates PKG and consequentially phosphorylates ERK to induce neurite outgrowth in PC12h cells.

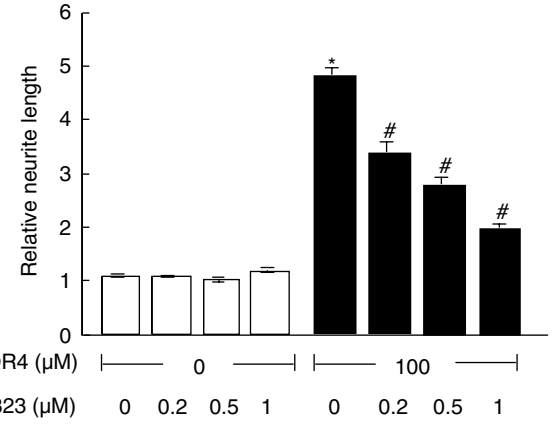

Figure 4 PKG inhibitor blocked NOR4-induced neurite outgrowth in PC12h cells. PC12h cells were pretreated with the indicated concentrations of KT5823 or its vehicle (0) for 15 min. Then, 100 μM NOR4 or its vehicle (0) was added to the culture. After 24 h, relative neurite length was evaluated as described in Methods. Both KT5823 and NOR4 were dissolved in dimethylsulfoxide. The final concentrations ($<0.2\%$ (v v^{-1})) of this solvent showed no cytotoxicity and neuritogenesis. The data shown represent the mean $\pm$ s.e. of three independent experiments. The mean value of neurite length was 12.4 ± 0.4 μm in vehicle-treated cells. $^{*}P<0.01$ *versus* vehicle alone and $^{\#}P<0.01$ *versus* NOR4 alone (ANOVA, Scheffe's test).

Discussion and conclusions

We first attempted an experiment to determine whether exogenous NO has neuritogenic activity using different types of NO donors. Treatment of PC12h cells with SNP, a metabolic NO donor (Kowaluk *et al*., 1992), significantly induced neurite outgrowth. However, since it is known that this donor generates not only NO but also cyanide (Tinker & Michenfelder, 1976), the range of efficacy might be limited. In fact, the medium preincubated with SNP in the absence of cells for 24 h had no neuritogenic activity and rather tended toward cytotoxicity in PC12h cells (data not shown).

Our second intention was to elucidate the mechanism of the neuritogenesis induced by NOR4 in PC12h cells. The NOR4-induced neurite outgrowth was extremely inhibited in the presence of carboxy-PTIO, ODQ, or KT5823, suggesting that NOR4 exerts its neuritogenic activity *via* NO-cGMP-PKG signaling pathway. Moreover, we observed that the NOR4-induced neuritogenesis was completely blocked by PD98059 and that NOR4 led to long-lasting ERK phosphorylation. Similar results were also observed in the 8-Br-cGMP-treated PC12h cells. Therefore, our data strongly suggested that the neurite outgrowth induced by NOR4 occurred *via* NO-cGMP-PKG signaling pathway-dependent ERK activation in PC12h cells. Furthermore, this mechanism was also observed in NGF-treated PC12h cells (Yamazaki *et al*., 2001; 2004), suggesting that the mechanism plays important role in neuronal differentiation. This suggestion is supported by other groups. That is, they also suggested that NO-cGMP-PKG pathway was involved in angiotensin II-induced neuronal differentiation in NG108-15 and PC12W cells (Gendron *et al*., 2002; Zhao *et al*., 2003). However, in this paper 8-Br-cGMP less induced neurite outgrowth than NOR4 even when PC12h cells were treated for 48 h, suggesting that NO could have other target molecule(s) besides sGC to induce neurite outgrowth.

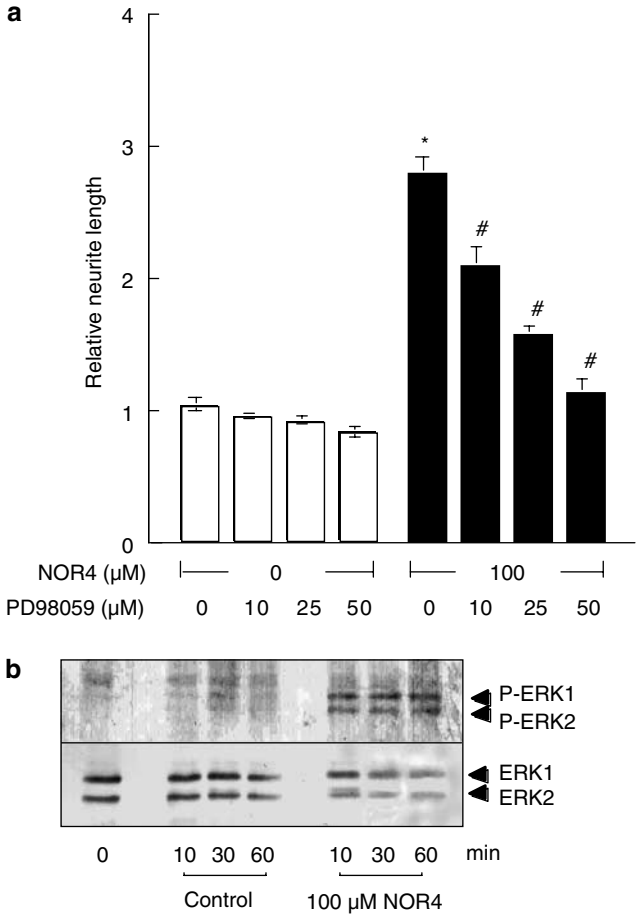

Figure 5 NOR4-induced neurite outgrowth through ERK activation in PC12h cells. (a) PC12h cells were pretreated with the indicated concentrations of PD98059 or its vehicle (0) for 60 min. Then, 100 μM NOR4 or its vehicle (0) was added to the culture. After 24 h, relative neurite length was evaluated as described in Methods. Both PD98059 and NOR4 were dissolved in dimethylsulfoxide. The final concentrations ($<0.2\%$ ($v\,v^{-1}$)) of this solvent showed no cytotoxicity and neuritogenesis. The data shown represent the mean$\pm$s.e. of three independent experiments. The mean value of neurite length was $17.0\pm0.9\,\mu$m in vehicle-treated cells. $^{*}P<0.01$ *versus* vehicle alone and $^{\#}P<0.01$ *versus* NOR4 alone (ANOVA, Scheffe's test). (b) PC12h cells were treated with 100 μM NOR4 or its vehicle (Control) for the indicated periods of time. The prepared cell extracts (10 μg protein/lane) were subjected to 10% SDS–PAGE and immunoblotted with antiserum against ERKs and activated ERKs (P-ERK). The blots at 0 min were from untreated cells. The data shown are typical of five independent experiments that indicated similar results.

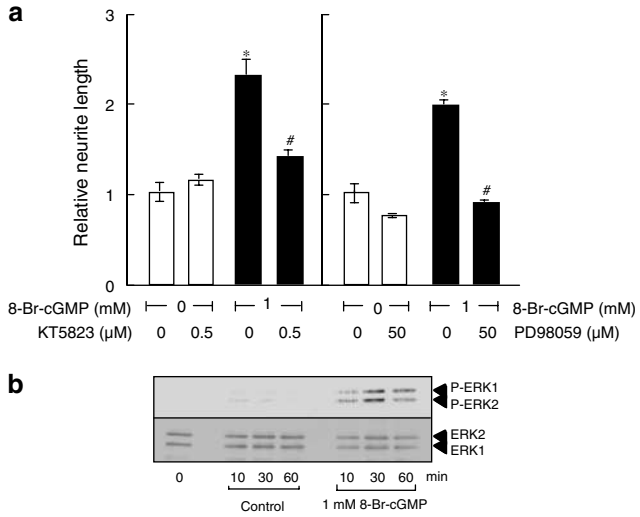

Figure 6 8-Br-cGMP-induced neurite outgrowth through PKG and ERK activation in PC12h cells. (a) PC12h cells were pretreated with 0.5 μM KT5823, 50 μM PD98059, or their vehicle (0) for 15 (for KT5823) or 60 min (for PD98059). Then, 1 mM 8-Br-cGMP or its vehicle (0) was added to the culture. After 48 h, relative neurite length was evaluated as described in Methods. KT5823 and PD98059 were dissolved in dimethylsulfoxide and 8-Br-cGMP was dissolved in PBS. The final concentrations ($<0.2\%$ ($v\,v^{-1}$)) of these solvents showed no cytotoxicity and neuritogenesis. The data shown represent the mean$\pm$s.e. of three independent experiments. The mean value of neurite length was $25.1\pm2.5\,\mu$m in vehicle-treated cells. $^{*}P<0.01$ *versus* corresponding values of vehicle alone and $^{\#}P<0.01$ *versus* corresponding values of NOR4 alone (ANOVA, Scheffe's test). (b) PC12h cells were treated with 1 mM 8-Br-cGMP or its vehicle (Control) for the indicated periods of time. The prepared cell extracts (10 μg protein/lane) were subjected to 10% SDS–PAGE and immunoblotted with antiserum against ERKs and activated ERKs (P-ERK). The blots at 0 min were from untreated cells. The data shown are typical of three independent experiments that indicated similar results.

For instance, it has been suggested that NO directly activates Ras by nitrosylation (Teng *et al*., 1999).

It has been reported that NO participates in the neuronal differentiation of parental PC12 cells by NGF (Peunova & Enikolopov, 1995; Poluha *et al*., 1997; Phung *et al*., 1999). Nevertheless, the direct effect of NO on neuritogenesis has never been reported in parental PC12 cells until now, although it has been demonstrated that NO itself does not induce neurite outgrowth but rather enhances NGF-induced neurite outgrowth (Hindley *et al*., 1997). However, we have just succeeded in observing NO-induced neurite outgrowth using PC12h cells but not parental PC12 cells. Similarly, it has also been demonstrated that SNP itself induces neurite outgrowth in NG108-15 and PC12W cells (Gendron *et al*., 2002; Zhao *et al*., 2003). On the other hand, we have also found that cGMP-PKG as well as NO contributes to neurite outgrowth in PC12h cells. It has been reported that a rise in intracellular cGMP levels is not involved in neuritogenesis in NGF-treated PC12 cells (Phung *et al*., 1999). Our results reveal a novel function for cGMP; it alone can induce neurite elongation *via* PKG activation in PC12h cells unlike in NGF-treated PC12 cells. In NG108-15 and PC12W cells, it has been reported that a membrane-permeable cGMP analog induces neurite outgrowth (Gendron *et al*., 2002; Zhao *et al*., 2003). In addition, Brown *et al*. (1999) have shown that bicyclic monoterpene diols induce neurite outgrowth *via* a cGMP-dependent pathway in PC12 cells. On the other hand, it has also been reported that NO has a cytoprotective effect in several cell types. For instance, NO donors delay the death of NGF- and serum-deprived PC12 cells and sympathetic neurons (Farinelli *et al*., 1996; Kim *et al*., 1999). Very recent studies have revealed that NO prevents 6-OHDA-induced apoptosis in PC12 cells (Ha *et al*., 2003) and that a low concentration of NO protects RAW264 cells against NO-induced cell death (Yoshioka *et al*., 2003). These reports suggest that such a protective effect of NO is mediated by a cGMP- or cGMP-PKG-dependent mechanism. Thus, we emphasize that the NO-cGMP-PKG signaling pathway might be an important pathway for neurotrophic actions such as differentiation and survival.

The present study has revealed that NO and cGMP caused sustained phosphorylation of ERK and that this event was involved in the neurite outgrowth induced by NO or cGMP in PC12h cells. The ERK phosphorylation mediated by NO has been described by several laboratories (Lander *et al*., 1996; Yan & Greene, 1998; Yun *et al*., 1999; Bauer *et al*., 2001). Thus, our results indicating that NO-induced neuronal differentiation *via* ERK activation are strongly supported by these reports. With respect to the relation between cGMP and ERK, Kim *et al*. (2000) reported that an NO donor phosphorylates both ERK and p38 MAPK in cardiomyocytes, suggesting that ERK phosphorylation is cGMP independent. In addition, it has been reported that ERK activation and NO-cGMP pathway are independent, but complementary, in neuritogenesis induced by angiotensin II in NG108-15 cells (Gendron *et al*., 2002). Thus, although the cells used in the experiments are different, our results suggest a novel mechanism in which NO-cGMP-PKG signaling pathway fundamentally involves ERK activation and plays an important role in the neuronal differentiation of PC12h cells.

Finally, we obtained a different result using PC12h cells from that of parental PC12 cells with respect to the responsiveness to NO donors. Hindley *et al*. (1997) and Phung *et al*. (1999) failed to observe NO donor-induced neurite outgrowth in parental PC12 cells. However, Hindley *et al*. (1997) have also indicated that NO donors elicit neurite outgrowth in hippocampal neurons prepared from embryonic day 17 mice. It seems that these contradictions might be attributed to the difference in amounts of NOS, especially nNOS, expression between these three cell types. Several groups have reported that the induction of nNOS is a key step in the induction of differentiation by NGF in PC12 cells (Hirsch *et al*., 1993; Sheehy *et al*. 1997; Phung *et al*., 1999). However, it has been demonstrated that untreated-PC12 cells do not express any types of NOS (Sheehy *et al*., 1997; Schonhoff *et al*., 2001), while untreated-PC12h cells express nNOS and iNOS slightly but significantly (Yamazaki *et al*., 2001). Moreover, we have detected that hippocampal neurons prepared from embryonic day 18 rats also express nNOS and eNOS (data not shown). Thus, we speculate that PC12h cells and primary neurons that endogenously express nNOS possess sufficient sGC and PKG required for NO-induced neuritogenesis, contrary to parental PC12 cells which do not express any types of NOS, although further analysis is still required. However, this speculation might be supported by Gendron *et al*. (2002) who reported that NG108-15 cells expressing nNOS extend neurites in response to NO donors and a membrane-permeable cGMP analog.

We conclude that NO itself can induce neurite outgrowth through NO-cGMP-PKG signaling pathway and that the signaling pathway consequently activates ERK in PC12h cells.

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