Induction of neurite outgrowth by α-phenyl-N-*tert*-butylnitrone through nitric oxide release and Ras-ERK pathway in PC12 cells

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Abstract

We have previously suggested that the spin trap agent, α-phenyl-N-*tert*-butylnitrone (PBN) induces neurite outgrowth through activation of the Ras-ERK pathway in PC12 cells. However, the chemical properties of PBN contributing to its biological function and the detailed mechanism for the activation of Ras by PBN remain unknown. Here, we demonstrate that the hydrophobic structure of PBN is related to the activation of Ras, by comparing with hydrophilic analogs of PBN. [^{14}C]-labeled PBN was found to localize in the lipid fraction and activate Ras indirectly. On the other hand, neurite outgrowth by PBN was inhibited by a nitric oxide (NO) scavenger. Moreover, the neurite outgrowth induced by PBN and the NO donor NOR4 was inhibited by the dominant negative Ras or MAPK/ERK inhibitor. Taken together, these results suggest that PBN is incorporated into the plasma membrane and induces neurite outgrowth in PC12 cells by activating the Ras-ERK pathway through NO release.

Keywords

Antioxidant, neurogenesis, nitric oxide

Introduction

The rat pheochromocytoma cell line PC12 has been used widely as a model for neuronal differentiation [1]. Neurotrophic factors such as nerve growth factor (NGF) and fibroblast growth factor (FGF) have been well established to differentiate PC12 cells into a sympathetic neuronal-like phenotype characterized by neurite outgrowth [1-3]. Numerous experiments to determine the mechanism involved in NGF-induced differentiation have been performed [4, 5]. NGF binds to and activates its high-affinity receptor, TrkA [6]. This activation sequentially induces phosphorylation of Shc, phospholipase C_γ (PLC_γ) and phosphatidylinositol 3-kinase (PI3K) [7]. The phosphorylation of Shc activates Ras and subsequent activation of the Raf/ERK signaling pathway leads to neuronal differentiation of PC12 cells [8, 9].

Recently, some proteins and peptides such as scoparone of a phytoalexin [10], cyrneine A of a novel cyathane diterpene [11], KNK437 of a heat shock protein inhibitor [12], a synthetic peptide ligand of neural cell adhesion molecule (ASKKPKRNIKA) [13] and octanoic acid [14] also have been reported to induce neurite outgrowth in PC12 cells. In our previous study, it was clarified that the nitron compound α-phenyl-N-*tert*-butylnitrone (PBN), which is widely used as a spin trap, induced neurite outgrowth in PC12 cells [15].

We also showed that PBN induced neurite outgrowth in PC12 cells through activation of the Ras/ERK pathway. Interestingly, PBN did not induce the activation of TrkA and subsequent activations of Shc, PI3K and PLC_γ unlike NGF. N-acetyl-L-cysteine (NAC) counteracted PBN-induced neurite outgrowth and the phosphorylation of ERK was prominently inhibited by NAC, dithiothreitol (DTT) and 2-mercaptoethanol [15]. Another report demonstrated that PBN up-regulated the content

of intracellular thiol and induced cell differentiation in a murine hematopoietic progenitor [16]. Based on these studies, it is suggested that PBN activates Ras directly or indirectly *via* a mechanism with a relationship to thiol residues. Lander et al. demonstrated that NO stimulated a Ras-related pathway through the S-nitrosylation of Cys-118 in Ras, using a cell-free system [17]. Furthermore, our preliminary experiment showed that the replacement to serine at Cys-118 (C118S) in Ras inhibited neurite outgrowth induced by PBN in PC12 cells. These results suggested the possibility that PBN also activates Ras through the cysteine residue in Ras, resulting in neurite induction of PC12 cells.

As an another mechanism of neurite induction of PC12 cells, Yamazaki et al. demonstrated that NO donors such as NOR4 induced PC12 cell neuritogenesis through activation of the cGMP-PKG pathway, indicating the ability of NO itself to induce neurite outgrowth [18]. Several studies demonstrated that PBN was decomposed at the C=N double bond and subsequently released NO *via* the cleavage of the C-N bond under oxidative conditions caused by UV irradiation and the Fenton reaction and under hyperthermic condition [19, 20]. These studies proposed the hypothesis that NO was released through the oxidative decomposition of PBN in PC12 cells.

On the other hand, PBN has been reported to be useful for preventing oxidative injury and age-related protein oxidation in the brain due to its ability to trap radicals [21, 22]. Combined with the neuritogenesis and the neuroprotective properties of PBN, it is thought to be important to compare the abilities of PBN and other spin trap compounds to induce neurite outgrowth and clarify the chemical nature required for this outgrowth. Thus, we first examined the neuritogenesis abilities of DMPO, PBN, more hydrophilic PBN analogs such as α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (POBN) and

N-*tert*-butyl-α-(2-sulfophenyl)-nitrone (S-PBN). Using synthesized [^{14}C]PBN, we examined the distribution of PBN and the interaction between PBN and Ras in PC12 cells. To investigate the possibility that NO released from PBN induces the activation of Ras, we examined whether the NO scavenger carboxy-PTIO inhibited neurite outgrowth induced by PBN. Furthermore, to clarify the responsibility of the Ras-ERK pathway for NO-induced neurite-outgrowth, we examined the effect of the NO donor NOR4 on neurite outgrowth of PC12 cells with and without inhibition of the Ras-ERK pathway by the MAPK/ERK inhibitor PD98059 or dominant-negative Ras (DNRas).

Materials and methods

Materials

PBN, POBN, S-PBN, 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and N-(*tert*-butyl)hydroxylamine were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Benzaldehyde-[ring-^{14}C] was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). The Ras antibody, Ras Assay Reagent (Raf-1 Ras binding domain (RBD) agarose), NGF, pUSEamp, and pUSEamp DNRas (S17N) were from Millipore Co. (Billerica, MA). Antibodies to ERK and phospho-ERK were from Cell Signaling Technology, Inc. (Danvers, MA). The actin antibody and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PD98059 was from Calbiochem-Novabiochem International Inc. (San Diego, CA). Carboxy-PTIO and NOR4 were from Dojindo Laboratories (Kumamoto, Japan). Lipofectamine^{TM} 2000 was from Invitrogen (Carlsbad, CA). pQBI25 was from Takara Shuzo (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Cell culture

PC12 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL/Invitrogen, Carlsbad, CA) supplemented with 10% horse serum and 5% fetal bovine serum (Filtron, Brooklyn, Australia) on poly-L-lysine-coated dishes at 37°C in 5% CO_2/95% air.

Neurite outgrowth

PBN, POBN, S-PBN, DMPO, NGF and NOR4 at the indicated concentrations were added to the culture medium, respectively. Neurite outgrowth of PC12 cells was estimated after incubation for 72 h in the presence of the drug. This incubation time is appropriate to evaluate neurite-inducible activity induced by these agents because we have demonstrated that many neurite outgrowth was observed (68.9% for 10 mM PBN and 98.8% for 50 ng/ml NGF of total cells) when cells were incubated for 72 h as shown in previous our experiments [15]. PD98059 or carboxy-PTIO was added 30 min before drug treatment. The percentage of cells with neurites extending at least 2 diameters from the cell body was counted.

SDS-PAGE and immunoblotting

PC12 cells were collected at the indicated periods after treatment with 10 mM PBN, 50 ng/ml NGF or 100 μM NOR4 and lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1% Triton X-100). Proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes (ADVANTEC Toyo, Tokyo, Japan). The membranes were probed with the indicated antibodies overnight at 4°C. The separated proteins were detected by a method using specific secondary antibodies with Perkin Elmer Western Lighting^{TM}, Chemiluminescence Reagent Plus (Perkin Elmer, Boston, MA). The bands were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Transient dominant negative Ras expression assay

DNRas(C118S) was developed using the site-directed mutagenesis method with

subcloned Ras cDNA. The entire Ras sequence and mutation were confirmed by sequence analysis. The transfection of PC12 cells with Ras(WT), DNRas(S17N) and DNRas(C118S) was performed using the Lipofectamine^{TM} 2000 reagent according to the manufacturer's instructions and the method described by Tsuji et al. [15]. For visualization, cells were cotransfected with pQBI25 vector encoding green fluorescent protein (rsGFP).

Activated Ras pull-down assay

GTP-binding Ras was measured following the protocol of the Ras activation assay kit. In brief, semi-confluent PC12 cells transfected with Ras(WT) and Ras(C118S) were treated with 10 mM PBN for 5 min or 100 μM NOR4 for 30 min. Cells were lysed in lysis buffer and the protein amount in each sample was adjusted to 1 μg/μl. For the preclear step, 20 μl of protein G-sepharose was added to 500 μl of each sample, which was then agitated for 30 min at 4°C. The supernatants were incubated with 5 μl of Raf-1 RBD agarose beads for 30 min at 4°C with gentle agitation, and then the beads were washed three times and resuspended in lysis buffer and a three-fold volume of Laemmli's sample buffer (0.625 M Tris–HCl [pH 6.8], 10% β-mercaptoethanol, 20% SDS, 20% glycerol, and 0.004% bromophenol blue). GTP-binding Ras protein was detected using immunoblot analysis.

Synthesis of [^{14}C]-labeled PBN

First, 120 mg of commercial benzaldehyde-[ring-^{14}C] (0.001 mol) and 100 mg of N-(*tert*-butyl)hydroxylamine (0.001 mol) were carefully mixed in 1 ml of ethanol. The mixture was dehydrated by stirring in the presence of molecular sieves for 96 h under

nitrogen gas. The recrystallization of PBN in hexane was performed by evaporation. To detect the synthesized [^{14}C]PBN, the ^{1}H NMR spectrum was examined with a Bruker ASX-300 NMR spectrometer (Bruker BioSpin GmbH, Silberstreifen, Germany) using tetramethylsilane (TMS) as an internal standard. In addition, we confirmed the spin trap ability of synthesized [^{14}C]PBN in the Fenton reaction by Fe^{2+} and H_2O_2 with a JOEL RE-1X ESR (Tokyo, Japan). The reaction mixture (200 µl) contained 7 µl $FeSO_4$ (10 mM), 7 µl H_2O_2 (1 M) and 100 µl PBN (100 mM). Spectrometer conditions were as follows: incident microwave power, 5 mW; modulation frequency, 100 kHz; field modulation amplitude, 0.2 mT; and scan range, 2.5 mT.

Fractionation of PC12 cells treated with [^{14}C]PBN

After incubation with medium containing 10 mM [^{14}C]PBN for 5 min, PC12 cells were collected and washed. The procedure of cell fractionation was according to the Schmidt-Thannhauser method using trichloroacetic acid (TCA) [23]. The ^{14}C radioactivity in the acid-soluble fraction, lipid fraction and insoluble fraction was measured with a liquid scintillation counter.

Immunoprecipitation

PC12 cells treated with 10 mM of PBN or [^{14}C]PBN for 5 min were lysed and precleared using protein G-Sepharose. The cell lysate was incubated with the anti-Ras antibody for 2 h at 4°C followed by protein G-Sepharose overnight at 4°C. Immunoprecipitates were washed three times with lysis buffer, separated by SDS–PAGE, transferred onto nitrocellulose membranes, and subjected to immunoblotting analysis using the anti-phospho-Ras antibody.

Statistical analysis

All results are expressed as the mean ± S.E. The variance ratio was estimated by the F-test and differences in means of groups were determined by Student's t-test or Welch's t-test. The minimum level of significance was set at $P<0.05$.

Results

The hydrophobic property of PBN was related to the induction of neurite outgrowth in PC12 cells

For the first experiment, we examined whether hydrophobicity was involved in PBN-induced neurite-outgrowth in PC12 cells. In addition to PBN, we prepared POBN, S-PBN and DMPO, the structures of which are shown in Figure 1A. PC12 cells were incubated with 10 mM PBN, 10 mM POBN, 10 mM S-PBN, 10 mM DMPO or 50 ng/ml NGF for 72 h. As shown in Figure 1B, no neurite outgrowth was found in control or DMPO-treated cells. Although PBN and NGF induced neurite outgrowth from almost all cells, only small numbers of neuronal differentiated cells were observed among cells treated with POBN and S-PBN (Figure 1B). The percentages of cells with neurites are presented in Figure 1C. PBN significantly induced neurite outgrowth in 79.7 ± 3.0% of PC12 cells at the same level as NGF (88.3 ± 1.4%). By contrast, the more hydrophilic PBN analogs POBN and S-PBN induced smaller numbers of cells with neurites than PBN, and their percentages were 50.1 ± 4.7% and 12.2 ± 1.8%, respectively. This meant that the hydrophobicity of PBN was related to the induction of neurite outgrowth.

PBN induced neurite outgrowth in PC12 cells through the activation of Ras-ERK pathway

To investigate the involvement of the Ras-ERK pathway in the induction of neurite outgrowth by PBN, we examined the effects of an inhibitor against MAPK/ERK kinase, PD98059, on neurite outgrowth induced by PBN in PC12 cells. PD98059 significantly inhibited neurite outgrowth induced by PBN and NGF compared to DMSO-treated cells

and the percentages were 27.0 ± 1.3% for PBN-treated cells and 38.8 ± 3.5% for NGF-treated cells (Figure 2A). In addition, the phosphorylation of ERK was observed at 5 min after PBN treatment as with NGF (Figure 2B). These results paralleled those of our previous study [15].

To clarify whether PBN affected the activatation of Ras, we also examined the expression of the activated form, GTP-binding Ras (GTP-Ras), by pull-down assay with Raf-1 RBD agarose. PC12 cells transiently transfected with Ras(WT) or DNRas(C118S) were treated with 10 mM PBN for 5 min. In Ras(WT)-transfected cells, PBN increased the ratio of Ras-GTP expression to total Ras expression up to 1.9-fold (Figure 2C). However, PBN induced weak activation of Ras in DNRas(C118S)-transfected cells.

To confirm the critical role of Ras in neurite outgrowth by PBN, we examined the effects of PBN and NGF on neurite induction in PC12 cells transfected with Ras(WT) or DNRas(S17N). Cells were cotransfected with a pQBI25 vector encoding rsGFP at 7:1 (DNRas/rsGFP) to enable the identification of cells transfected with these constructs. By observing only rsGFP-positive cells, the levels of induction of neurite outgrowth by PBN and NGF in cells transfected with the control vector pUSEamp were confirmed to be 54.3 ± 12.9% and 77.7 ± 4.0%, respectively (Figure 3). By contrast, the percentages of neurite outgrowth in PBN- and NGF-stimulated cells transfected with DNRas(S17N) were significantly decreased to 32.0 ± 2.0% and 44.7 ± 8.5%, respectively. In addition, the transfection of Ras(WT) induced neurite outgrowth in about 45% of the PC12 cells without any treatment and the rates were increased to 71.0 ± 1.7% and 84.7 ± 5.5% by PBN and NGF, respectively. These results demonstrated that PBN induced neurite outgrowth *via* the ERK pathway by the activation of Ras in a

manner similar to that NGF.

PBN was localized in plasma membrane and was not directly associated with Ras

To examine the distribution of PBN and the linkage between PBN and Ras in PC12, we synthesized [^{14}C]PBN as described in Materials and Methods. To confirm the chemical nature of the product synthesized as [^{14}C]PBN, we performed NMR spectrometry. As shown in Figure 4A, two apparent NMR lines at 1.6 ppm and 8.0 ppm corresponding to the ^{1}H chemical shift of the methyl residue and benzene ring were observed and the NMR spectrum obtained from the synthesized compound completely coincided with that from a commercial PBN. Furthermore, to confirm accurate synthesis of [^{14}C]PBN, we tested the scavenging ability of this synthesized [^{14}C]PBN against the $^{\cdot}$OH radical using the ESR method. As shown in Figure 4A, the formation of a PBN/$^{\cdot}$OH adduct was clearly observed. The hyperfine constants of the ESR spectrum were equal to A_N = 1.53 mT and $A_{H\beta}$ =0.25 mT and were in good agreement with those reported for the spin adduct of the $^{\cdot}$OH radical with PBN [24]. This result indicated that [^{14}C]PBN was accurately synthesized with similar spin trapping ability to a commercial one.

Next, we performed cell fractionation assay using [^{14}C]PBN to determine the distribution of PBN in PC12 cells. As mentioned in Materials and Methods, PC12 cells treated with [^{14}C]PBN were separated into acid-soluble, lipid and insoluble fractions and then the ^{14}C radioactivity levels in them were measured (Figure 4B). The radioactivity in lipid fraction was higher (24.7 ± 13.9 cpm) than in the other fractions (7.6 ± 3.6 cpm for the acid-soluble fraction and 4.3 ± 1.2 cpm for the insoluble fraction).

To investigate whether PBN bound to Ras protein directly, PC12 cells were

treated with [^{14}C]PBN for 5 min and then Ras protein was immunoprecipitated using a specific antibody. Immunoblot analysis confirmed the expression of Ras in [^{14}C]PBN-treated cells (Figure 4C). When the ^{14}C radioactivity in this immunoprecipitate for Ras was measured, less was observed (2.5 cpm) than in the non-immunoprecipitate control (19.0 cpm) (Figure 4D). These results suggested that PBN was localized in the lipid fraction and was not associated with Ras protein directly.

Inhibition of neurite outgrowth in PC12 cells by NO scavenger

Recently, it has been reported that NO itself has the ability to induce neurite outgrowth through the NO-cGMP-PKG signaling pathway, as demonstrated by an experiment using an NO donor [18]. On the basis of this report, we examined whether carboxy-PTIO, known as an NO scavenger [25], could affect the abilities of PBN and NGF to induce neurite outgrowth. As shown in Figure 5A, pretreatment with 50 μM carboxy-PTIO obviously inhibited PBN-induced neurite outgrowth. When various concentrations of carboxy-PTIO were used for pretreatment 30 min before PBN treatment, PBN-induced neurite-outgrowth was attenuated by PBN dose-dependently (Figure 5B). However, carboxy-PTIO could not inhibit NGF-induced neurite outgrowth. These results suggested that the mechanism of neurite outgrowth induced by PBN was related to NO, unlike that induced by NGF.

Neurite outgrowth induced by NO donor in PC12 cells

Next, we examined whether neurite outgrowth could be induced by NO itself. For NO treatment, we used NOR4, which is well established to be an NO donor [26, 27]. Figure 6 shows the percentages of neurite outgrowth from PC12 cells treated with 0-100 μM

NOR4 for 72 h. NOR4 induced neurite outgrowth dose dependently without any toxicity, and the percentage in cells treated with 100 μM NOR4 was 46.8 ± 8.5%.

NO-induced neurite outgrowth is dependent on Ras-ERK pathway

To clarify whether NO-induced neurite outgrowth was involved in the Ras-ERK pathway, like that induced by PBN, we evaluated the rate of neurite outgrowth induced by NOR4 in PC12 cells transiently transfected with DNRas(S17N) or Ras(WT). As shown in Figure 7A, the induction of neurite outgrowth in cells transfected with DNRas(S17N) was decreased to 5.3 ± 1.3% compared to cells with the control vector (32.5 ± 6.2%). The transfection of Ras(WT) increased the rate to 91.0 ± 0.9%. This tendency was similar to that in PBN-treated cells.

Furthermore, to investigate the activation of the Ras-ERK pathway in NOR4-treated cells, we examined the expression of ERK, phosphorylated ERK and Ras by immunoblotting analysis and Ras-GTP by pull-down assay. As shown in Figure 7B, the phosphorylation of ERK in PC12 cells was increased by NOR4 treatment and the ratio of p-ERK/actin at 10 min was 1.5 compared to that of control. This up-regulation was completely attenuated by pretreatment with 50 μM PD98059 for 30 min. Pull-down assay showed that NOR4 treatment increased the amount of Ras-GTP in cells transiently transfected with Ras(WT); however, NOR4-induced formation of Ras-GTP was not observed in Ras-deactivated cells by transfection of DNRas(C118S) (Figure 7C). These results paralleled those in the case of PBN (Figures 2B and 2C), suggesting that activation of the Ras-ERK pathway was responsible for the induction of neurite outgrowth by NO as well as PBN.

Discussion

The signaling pathways involved in neuronal differentiation have been extensively studied in PC12 cells. As the first reaction, NGF stimulates TrkA receptor tyrosine kinase in PC12 cells [28]. The activation of TrkA induces the phosphorylation of PLC, PI3-kinase, and Shc [7, 29]. This leads the recruitment to the membrane of a complex of adaptor proteins Grb-2 and SOS, thereby stimulating transient activation of Ras [4, 30]. The activation of Ras causes activation of the Ras-ERK cascade, resulting in neurite outgrowth [31, 32]. Our previous study demonstrated that PBN induced neurite outgrowth through activation of the Ras-ERK pathway and PKC [15]. Interestingly, this induction was not associated with the activation of TrkA and subsequent activation of Shc, PI3-kinase, and PLC, unlike that by NGF. However, the detailed mechanism for PBN-induced activation of Ras has been unclear.

In the present study, neurite outgrowth of PC12 cells induced by PBN was significantly inhibited by pretreatment with the MAPK/ERK inhibitor PD98059 (Figure 2A). Immunoblot analysis demonstrated that the expression of phosphorylated ERK increased and reached a peak at 5 min after PBN treatment (Figure 2B). Furthermore, pull-down assay using Raf-1 RBD agarose showed that PBN up-regulated the expression of Ras-GTP in Ras(WT)-transfected PC12 cells, but this up-regulation was attenuated in dominant-negative Ras-mutated Cys-118 to serine (Figure 2C). The transfection of Ras(WT) enhanced PBN-induced neurite outgrowth (Figure 3). In contrast, the transfection of DNRas(S17N) with low affinity to GTP [33] inhibited PBN-induced neurite outgrowth. These results suggested that PBN-induced neurite outgrowth was mediated by activation of the Ras-ERK pathway and the mutation of Cys-118 inhibited the GDP-GTP reaction induced by PBN. Since PBN activates the

Ras-ERK pathway without the activation of TrkA, and subsequently PI3K and PLCγ [15], this suggested that PBN activated Ras, especially at the Cys-118 residue in Ras, directly or indirectly.

To elucidate the mechanism for PBN-induced activation of the Ras-ERK pathway, we synthesized [^{14}C]-labeled PBN, which was confirmed by proton NMR spectrometry and ESR spectrometry (Figure 4A). Among the cell fractions separated by the Schmidt-Thannhauser method, the strongest [^{14}C] radioactivity was observed in the lipid fraction (Figure 4B), indicating the localization of PBN in the plasma membrane. We also demonstrated that more hydrophilic PBN analogs, POBN and S-PBN had weak abilities to induce neuritogenesis compared to that of PBN (Figures 1B and 1C). Therefore, the hydrophobicity may be related to neurite outgrowth due to localization in the plasma membrane. Moreover, immunoprecipitation assay for Ras from [^{14}C]PBN-treated cells demonstrated that PBN was not directly associated with Ras protein (Figure 4D), suggesting indirect interaction between PBN and Ras. As another reason why neuritogenesis ability was dependent on the chemical structure of the spin trapping agent, there is a possibility that neuritogenesis ability is associated with $^{\cdot}$OH radical spin trapping efficacy of each spin trap agent. However, Kim et al. [34] and William et al. [35] reported that trapping efficiency of PBN against $^{\cdot}$OH radicals is lower than that of POBN or S-PBN in cell free system. Therefore, these reports indicated that the neuritogenesis induced by nitrone spin trapping agents was associated with their hydrophobicity but not their hydroxyl radical scavenging ablity.

Here, the question how PBN stimulates Ras indirectly remains. As one possibility, several studies reported that NO was generated from PBN under certain oxidative conditions [19, 20]. NO was also reported to bind to and S-nitrosylate

Cys-118 residue in Ras, activating the ERK cascade [17]. In the present study, pretreatment with the NO scavenger carboxy-PTIO inhibited PBN-induced neurite outgrowth in a dose-dependent manner, but not NGF-induced neurite-outgrowth (Figure 5). These facts suggested that NO was produced from PBN under unknown intracellular oxidative circumstances in PC12 cells exposed to PBN and involved in activation of Ras to lead to neuritogenesis in PC12 cells exposed to PBN. Whereas NGF induced neuritogenesis-related Ras/ERK cascade through a well-known signal transduction molecules such as TrkA, Shc, Grb-2, PLC_{γ} and PI3K but not NO [4, 7, 28-32]. Furthermore, our results showed the ability of the NO donor NOR4 to induce neuritogenesis (Figure 6), supporting a previous report [18]. In addition, DNRas(S17N) transfection inhibited NOR4-induced neurite outgrowth (Figure 7A). NOR4 also induced the activation of both ERK and Ras in PC12 cells (Figures 7B and 7C). These results paralleled that of PBN-induced neuritogenesis, suggesting the possibility that NO released from PBN was involved in neurite outgrowth. In this study, the treatment of carboxy-PTIO, an NO scavenger, reduced PBN-induced neurite outgrowth from 75% to 30% of PC12 cells, indicating that NO-related neurite outgrowth in PC12 cells exposed to 10 mM PBN was at least 45% of total cells as fraction inhibited by carboxy-PTIO (Figure 5B). On the other hand, the treatment of 100 μM NOR4 induced neurite outgrowth to about 47% of total PC12 cells (Figure 6) and the decomposition of NOR4 was known to produce same amount of NO [36]. Since fraction of NO-related neurite outgrowth in PBN-treated PC12 cells was equivalent to that of NOR4-induced neurite outgrowth, it was assumed that the amount of NO accumulated in PC12 cells exposed 10 mM PBN reached to at least 100 μM. However, this NO release from PBN in PC12 cells may be very slow in intracellular oxidative condition, because the other

NOR analogs such as NOR2 and NOR3, which release NO rapidly compared to NOR4, have been reported to have severe toxic effects on cells [26].

In conclusion, PBN localized in the plasma membrane stimulates the Ras-ERK pathway through NO release and S-nitrosylation of Cys-118 in Ras protein, resulting in neurite outgrowth in PC12 cells.

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Figures & Legends

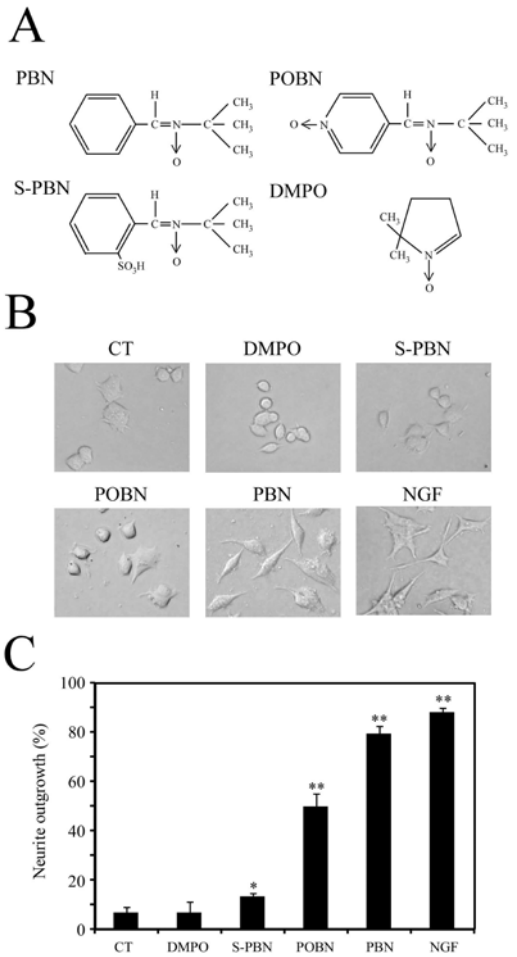

Figure 1.

Neurite outgrowth induced in PC12 cells by PBN analogs. (A) Structures of PBN analogs and DMPO. Representative images obtained by phase-contrast microscopy (B) and the percentages (C) of neurite outgrowth in cells induced by DMPO, S-PBN, POBN, PBN and NGF. PC12 cells were stimulated by the indicated concentration of each drug for 72 h. Each value is the mean ± S.E. of about 200 cells obtained from three independent experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* control.

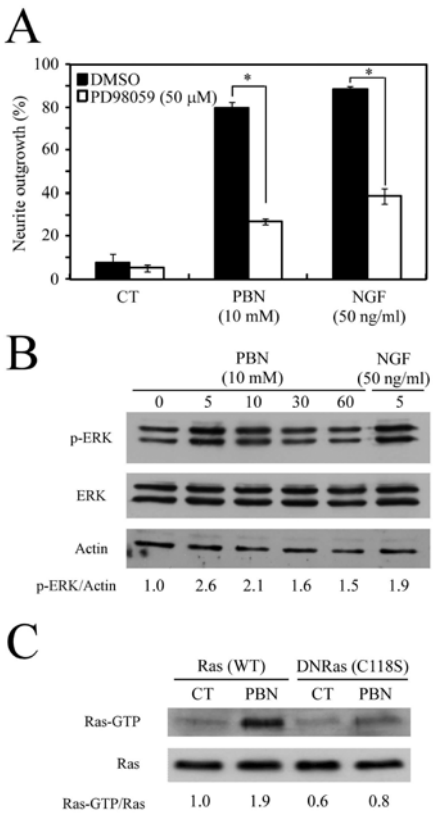

Figure 2.

Involvement of the activation of ERK and Ras in PBN-induced neurite outgrowth. (A) Inhibitory effect of PD98059 on neurite outgrowth induced by PBN or NGF. PC12 cells were pretreated with 50 μM PD98059 for 30 min before the exposure to PBN and NGF. Each value is the mean ± S.E. *, $p < 0.01$. (B) Time-course of the ERK activation by PBN. After the treatment of PC12 cells with PBN or NGF for the indicated times, immunoblots for p-ERK, ERK and actin were performed. Quantification of bands was performed using Image J software and the ratio of p-ERK against actin is presented. (C) The activation of Ras induced by PBN was inhibited by the transfection of DNRas. PC12 cells were transiently transfected with Ras(WT) or DNRas(C118S), followed by exposure to PBN. The expression of Ras-GTP was detected by pull-down assay. The

total expression of Ras was also detected by immunoblotting. The ratio of Ras-GTP to Ras is shown at the bottom.

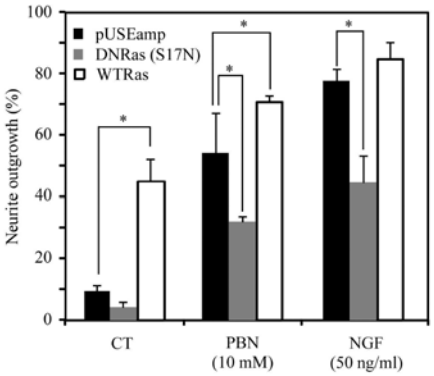

Figure 3.

Inhibition of PBN-induced neurite outgrowth by transfection of DNRas. After the transient cotransfection of pUSEamp, DNRas(S17N) or Ras(WT) with rsGFP vector, PC12 cells were stimulated with 10 mM PBN or 50 ng/ml NGF for 72 h. The percentage of rsGFP-positive cells with neurites was determined. Each value is the mean ± S.E. of about 100 cells obtained from three independent experiments. *, $p < 0.01$ *versus* pUSEamp-transfected control.

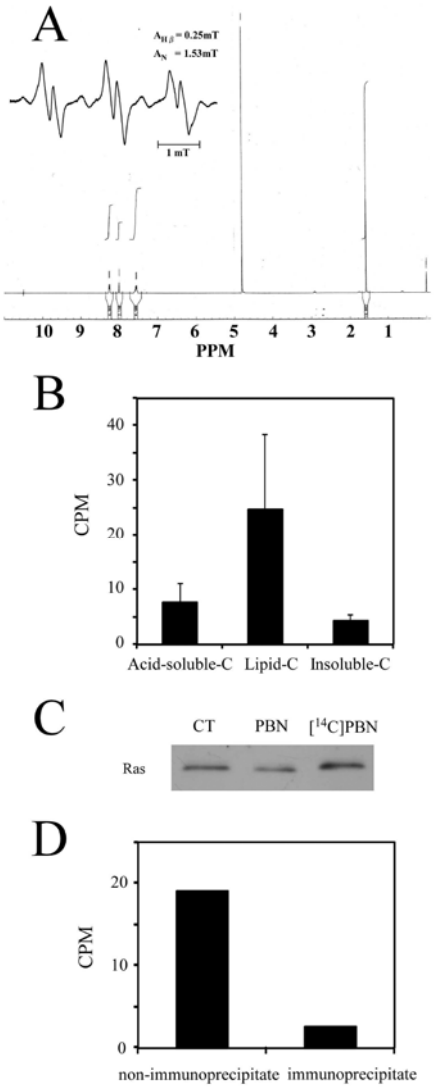

Figure 4.

Distribution of PBN in PC12 cells and interaction between PBN and Ras. (A) The synthesis of [^{14}C]PBN. [^{14}C]PBN synthesized as described in Materials and Methods was confirmed by NMR spectrometry (*center*) and ESR spectrometry (*left top*). The ^{1}H chemical shifts of the methyl residue and benzene ring in the NMR spectrum and the formation of a PBN/˙OH adduct in the ESR spectrum were observed. (B) Distribution of [^{14}C]PBN in PC12 cells. PC12 cells treated with [^{14}C]PBN were fractionated by the

Schmidt-Thannhauser method. Then ^{14}C radioactivity levels in the acid-soluble fraction, lipid fraction and insoluble fraction were measured with a liquid scintillation counter. Data are presented as the mean ± S.E. (C) Induction of Ras protein by [^{14}C]PBN. The expression of Ras was determined by immunoblot analysis using the cell lysate from PC12 cells treated with PBN or [^{14}C]PBN. (D) Indirect interaction between PBN and Ras. PC12 cells were treated with [^{14}C]PBN for 5 min and Ras protein was immunoprecipitated using its specific antibody. The ^{14}C radioactivity in this immunoprecipitate for Ras was measured compared to the supernatant as a control non-immunoprecipitate.

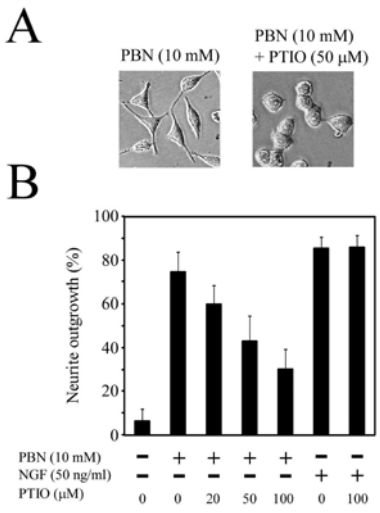

Figure 5.

Inhibition of PBN-induced neurite outgrowth by NO scavenger. After pretreatment with 50 μM carboxy-PTIO for 30 min, PC12 cells were stimulated with 10 mM PBN or 50 ng/ml NGF for 72 h. (A) Representative images were obtained by phase-contrast microscopy. (B) Inhibition of PBN-induced neurite outgrowth by carboxy-PTIO in a dose-dependent manner. PC12 cells were pretreated with the indicated concentrations of carboxy-PTIO for 30 min before the exposure to PBN or NGF. Each value is the mean ± S.E.

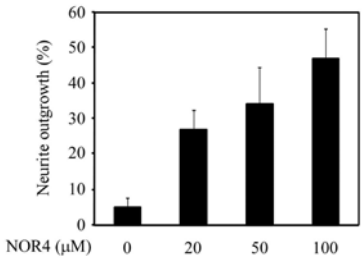

Figure 6.

Dose-dependent response of neurite outgrowth induced by the NO donor NOR4 in PC12 cells. PC12 cells were exposed to the indicated concentrations of NOR4 for 72 h. Each value is the mean ± S.E.

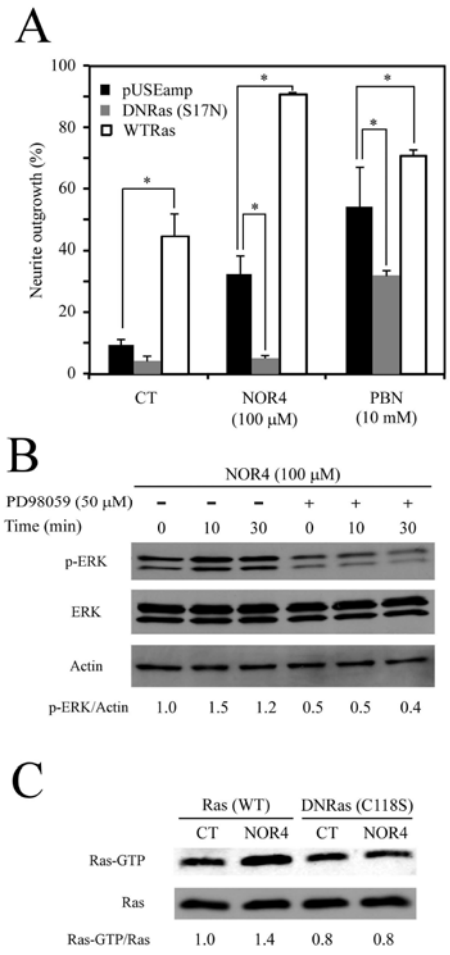

Figure 7.

NOR4 induced neurite outgrowth in PC12 cells through the Ras-ERK pathway. (A) Inhibition of NOR4-induced neurite outgrowth by transfection of DNRas. After the transient cotransfection of pUSEamp, DNRas(S17N) or Ras(WT) with rsGFP vector, PC12 cells were stimulated with 100 μM NOR4 or 10 mM PBN for 72 h. The percentage of rsGFP-positive cells with neurites was determined. Each value is the mean ± S.E. of about 100 cells obtained from three independent experiments. *, $p < 0.01$ *versus* pUSEamp-transfected control. (B) Inhibitory effect of PD98059 on NOR4-induced ERK-phosphorylation. PC12 cells were pretreated with 50 μM

PD98059 for 30 min before NOR4 treatment. After the treatment with 100 μM NOR4 for the indicated times, PC12 cells were collected and lysed. Immunoblotting for p-ERK, ERK and actin was performed. Quantification of bands was performed using Image J software and the ratio of p-ERK against actin is presented. (C) The activation of Ras induced by NOR4 was inhibited by the transfection of DNRas. PC12 cells were transiently transfected with Ras(WT) or DNRas(C118S), followed by exposure to NOR4. The expression of Ras-GTP was detected by pull-down assay. The total expression of Ras was also detected by immunoblotting. The ratio of Ras-GTP to Ras is shown at the bottom.

References

[1] Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A 1976;73:2424-2428.

[2] Cowley S, Paterson H, Kemp P, Marshall CJ. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 1994;77:841-852.

[3] Burstein DE, Blumberg PM, Greene LA. Nerve growth factor-induced neuronal differentiation of PC12 pheochromocytoma cells: lack of inhibition by a tumor promoter. Brain Res 1982;247:115-119.

[4] Patapoutian A, Reichardt LF. Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 2001;11:272-280.

[5] Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 2003;72:609-642.

[6] Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 1991;252:554-558.

[7] Obermeier A, Bradshaw RA, Seedorf K, Choidas A, Schlessinger J, Ullrich A. Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC gamma. Embo J 1994;13:1585-1590.

[8] Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, Bar-Sagi D, Margolis B, Schlessinger J. Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature 1993;363:85-88.

[9] Rozakis-Adcock M, McGlade J, Mbamalu G, Pelicci G, Daly R, Li W, Batzer A, Thomas S, Brugge J, Pelicci PG, Schlessinger J, Pawson T. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature 1992;360:689-692.

[10] Yang YJ, Lee HJ, Choi DH, Huang HS, Lim SC, Lee MK. Effect of scoparone on neurite outgrowth in PC12 cells. Neurosci Lett 2008;440:14-18.

[11] Obara Y, Hoshino T, Marcotullio MC, Pagiotti R, Nakahata N. A novel cyathane diterpene, cyrneine A, induces neurite outgrowth in a Rac1-dependent mechanism in PC12 cells. Life Sci 2007;80:1669-1677.

[12] Koike T, Uno S, Ishizawa M, Takahashi H, Ikeda K, Yokota S, Makishima M. The heat shock protein inhibitor KNK437 induces neurite outgrowth in PC12 cells. Neurosci Lett 2006;410:212-217.

[13] Ronn LC, Doherty P, Holm A, Berezin V, Bock E. Neurite outgrowth induced by a synthetic peptide ligand of neural cell adhesion molecule requires fibroblast growth factor receptor activation. J Neurochem 2000;75:665-671.

[14] Kamata Y, Shiraga H, Tai A, Kawamoto Y, Gohda E. Induction of neurite outgrowth in PC12 cells by the medium-chain fatty acid octanoic acid. Neuroscience 2007;146:1073-1081.

[15] Tsuji M, Inanami O, Kuwabara M. Induction of neurite outgrowth in PC12 cells by alpha -phenyl-N-tert-butylnitron through activation of protein kinase C and the Ras-extracellular signal-regulated kinase pathway. J Biol Chem 2001;276:32779-32785.

[16] Kashiwakura I, Kuwabara M, Murakami M, Hayase Y, Takagi Y. Effects of alpha-phenyl N-tert-butylnitrone, a spin trap reagent, on the proliferation of

murine hematopoietic progenitor cells in vitro. Res Commun Mol Pathol Pharmacol 1997;98:67-76.

[17] Lander HM, Milbank AJ, Tauras JM, Hajjar DP, Hempstead BL, Schwartz GD, Kraemer RT, Mirza UA, Chait BT, Burk SC, Quilliam LA. Redox regulation of cell signalling. Nature 1996;381:380-381.

[18] Yamazaki M, Chiba K, Mohri T. Fundamental role of nitric oxide in neuritogenesis of PC12h cells. Br J Pharmacol 2005;146:662-669.

[19] Saito K, Yoshioka H, Kazama S, Cutler RG. Release of nitric oxide from a spin trap, N-tert-butyl-alpha-phenylnitrone, under various oxidative conditions. Biol Pharm Bull 1998;21:401-404.

[20] Cui ZG, Kondo T, Matsumoto H. Enhancement of apoptosis by nitric oxide released from alpha-phenyl-tert-butyl nitrone under hyperthermic conditions. J Cell Physiol 2006;206:468-476.

[21] Tsuji M, Inanami O, Kuwabara M. Neuroprotective effect of alpha-phenyl-N-tert-butylnitrone in gerbil hippocampus is mediated by the mitogen-activated protein kinase pathway and heat shock proteins. Neurosci Lett 2000;282:41-44.

[22] Carney JM, Starke-Reed PE, Oliver CN, Landum RW, Cheng MS, Wu JF, Floyd RA. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-tert-butyl-alpha-phenylnitrone. Proc Natl Acad Sci U S A 1991;88:3633-3636.

[23] Schmidt G, Thannhauser SJ. A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues.

J Biol Chem 1945;161:83-89.

[24] Pou S, Ramos CL, Gladwell T, Renks E, Centra M, Young D, Cohen MS, Rosen GM. A kinetic approach to the selection of a sensitive spin trapping system for the detection of hydroxyl radical. Anal Biochem 1994;217:76-83.

[25] Akaike T, Yoshida M, Miyamoto Y, Sato K, Kohno M, Sasamoto K, Miyazaki K, Ueda S, Maeda H. Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/.NO through a radical reaction. Biochemistry 1993;32:827-832.

[26] Yamamoto T, Yuyama K, Nakamura K, Kato T, Yamamoto H. Kinetic characterization of the nitric oxide toxicity for PC12 cells: effect of half-life time of NO release. Eur J Pharmacol 2000;397:25-33.

[27] Kato M, Nishino S, Ohno M, Fukuyama S, Kita Y, Hirasawa Y, Nakanishi I, Takasugi H, Sakane K. New reagents for controlled release of nitric oxide. Structure-stability relationships. Bioorg Med Chem Lett 1996;6:33-38.

[28] Jing S, Tapley P, Barbacid M. Nerve growth factor mediates signal transduction through trk homodimer receptors. Neuron 1992;9:1067-1079.

[29] Ashcroft M, Stephens RM, Hallberg B, Downward J, Kaplan DR. The selective and inducible activation of endogenous PI 3-kinase in PC12 cells results in efficient NGF-mediated survival but defective neurite outgrowth. Oncogene 1999;18:4586-4597.

[30] Xing J, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 1996;273:959-963.

[31] Vaudry D, Stork PJ, Lazarovici P, Eiden LE. Signaling pathways for PC12 cell

differentiation: making the right connections. Science 2002;296:1648-1649.

[32] Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179-185.

[33] John J, Rensland H, Schlichting I, Vetter I, Borasio GD, Goody RS, Wittinghofer A. Kinetic and structural analysis of the Mg(2+)-binding site of the guanine nucleotide-binding protein p21H-ras. J Biol Chem 1993;268:923-929.

[34] Kim S, de AVGV, Bouajila J, Dias AG, Cyrino FZ, Bouskela E, Costa PR, Nepveu F. Alpha-phenyl-N-tert-butyl nitrone (PBN) derivatives: synthesis and protective action against microvascular damages induced by ischemia/reperfusion. Bioorg Med Chem 2007;15:3572-3578.

[35] Williams HE, Claybourn M, Green AR. Investigating the free radical trapping ability of NXY-059, S-PBN and PBN. Free Radic Res 2007;41:1047-1052.

[36] Kita Y, Hirasawa Y, Fukuyama S, Ohkubo K, Kato Y, Takamatsu H, Ohno M, Nishino S, Kato M, Seki J. Oral biological activities of spontaneous nitric oxide releasers are accounted for by their nitric oxide-releasing rates and oral absorption manners. J Pharmacol Exp Ther 1996;276:421-425.