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Nerve growth factor-induced Neuronal Differentiation Requires Generation of Rac1-regulated Reactive Oxygen Species*

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Kazumi Suzukawa‡, Koichi Miura‡, Junji Mitsushita‡, James Resau¶, Kunita Hiron˚, Ronald Crystal‡‡, and Tohru Kamata‡§ ‡‡

From the ‡‡Science Applications International Corporation/Frederick, NCI-Frederick Cancer Research and Development Center, National Institutes of Health, Frederick, Maryland 21702, the §Department of Molecular Biology and Biochemistry, Shinshu University School of Medicine, Matsumoto, Nagano 390-8621, Japan, the ¶Analytical Microscopy Laboratory, Advanced Bioscience Laboratories-Basic Research Program, NCI-Frederick Cancer Research and Development Center, National Institutes of Health, Frederick, Maryland 21702, the §Biomedical Research Institute, Kureha Chemical, Tokyo 160, Japan, and **Cornell University Medical College, New York, NY 10021

Nerve growth factor (NGF) stimulation of pheochromocytoma PC12 cells transiently increased the intracellular concentration of reactive oxygen species (ROS). This increase was blocked by the chemical antioxidant N-acetylcysteine and a flavoprotein inhibitor, diphenylene iodonium. NGF responses of PC12 cells, including neurite outgrowth, tyrosine phosphorylation, and AP-1 activation, was inhibited when ROS production was prevented by N-acetylcysteine and diphenylene iodonium. The expression of dominant negative RacN17 blocked induction of both ROS generation and morphological differentiation by NGF. The ROS produced appears to be H2O2, because the introduction of catalase into the cells abolished NGF-induced neurite outgrowth, ROS production, and tyrosine phosphorylation. These results suggest that the ROS, perhaps H2O2, acts as an intracellular signal mediator for NGF-induced neuronal differentiation and that NGF-stimulated ROS production is regulated by Rac1 and a flavoprotein-binding protein similar to the phagocytic NADPH oxidase.

The production of ROS such as superoxide (O2·−) and hydrogen peroxide (H2O2) was observed in a number of cells stimulated with cytokines such as transforming growth factors-β1 (6, 7), interleukin-1 (8), and tumor necrosis factor α (9) or peptide growth factors such as platelet-derived growth factor (PDGF) (10) and epidermal growth factor (EGF) (11). H2O2 has been shown to mediate PDGF-induced cellular DNA synthesis of rat vascular smooth muscle cells (10). Ras-dependent cell growth requires generation of the O2·− free radical through a pathway involving Rac1 (12).

Although the role of ROS has been extensively studied in mitogenesis, inflammation, and apoptosis (1), little is known about its functional role in the differentiation process. The differentiation process in the nervous system is regulated by the action of differentiation and growth factors including NGF. NGF induces the growth arrest of PC12 cells and promotes their differentiation into sympathetic neuron-like cells (13). NGF binding to its receptor tyrosine kinase, TrkA, initiates various molecular interactions including tyrosine phosphorylation of proteins and the activation of the Ras/Raf/MEK/MAPK pathway (14, 15). NGF induces the production of reactive nitric oxide (NO), and NO is required for NGF-induced cytoskeleton and differentiation (16), suggesting that free radical molecules such as NO and ROS may exert a regulatory role in certain types of cellular differentiation. In the current study, we focused on the role of ROS and a small GTP-binding protein, Rac1, in the NGF-induced neuronal differentiation.

**Experimental Procedures**

Measurement of Intracellular ROS—PC12 cells were cultured in DMEM supplemented with 10% horse serum and 5% fetal bovine serum. PC12 cells were plated in a poly-L-lysine-coated 12-well plate (Corning Glass) and serum-starved in 0.5% horse serum and 0.25% fetal bovine serum for 12–15 h. After rinsing with DMEM lacking phenol red, cells were loaded with 2′,7′-dichlorofluorescein diacetate (DCFDA; 5 μg/ml, Molecular Probes) for 10 min at 37 °C and then the indicated amounts of NGF (Upstate Biologicse) were added. The DCF fluorescence intensity was measured by a CytoFlour plate reader (PerSeptive Biosystems) (excitation wavelength, 485 nm; emission wavelength, 530 nm).

Enzyme Inhibitors and Western Blotting—PC12 cells were pretreated with indicated amounts of NAC (Sigma) or DPI (Molecular Probes) for 2 h, and NGF (50 ng/ml) was added to the cells for 48 h. Cells were fixed in 4% paraformaldehyde for 20 min, and cell morphology was observed. Serum-starved 6–24 cells (PC12 cells overexpressing TrkA) were pre-treated with NAC or DPI for 2 h and then unstimulated or stimulated with NGF for 5 min. Cells were disrupted in the buffer (25 mM Tris-Cl, pH 7.5, 0.2% Nonidet P-40, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml pepstatin A). Lysates were analyzed by immuno-blotting with anti-phosphotyrosine antibody (4G10).

Transfection—PC12 cells were transfected with pcDNA-wt Rac1, pcDNA-Rac1N17, pcDNA-Rac1V12, and empty pcDNA vector together with pcDNA plasmids using LipofectAMINE (Gibco BRL).

Enzyme Inhibitors—PC12 cells were transfected with pcDNA-wt Rac1, pcDNA-Rac1N17, pcDNA-Rac1V12, and empty pcDNA vector together with pcDNA plasmids using LipofectAMINE (Gibco BRL).

DCFDA, 2′,7′-dichlorofluorescein diacetate; DCF, dichlorofluorescein; NAC, N-acetylcysteine; DPI, diphenylene iodonium; wt, wild-type; NBT, nitro blue tetrazolium; EMSA, electrophoretic mobility shift assay; PTPros(a), protein-tyrosine phosphatase(s); PAGE, polyacrylamide gel electrophoresis.

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‡‡ To whom correspondence should be addressed: Dept. of Molecular Biology and Biochemistry, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto, Nagano 390-8621, Japan. Tel.: 81-263-37-2603; Fax: 81-263-37-2604; E-mail: kamata@sch.md.shinshu-u.ac.jp.

¶ The abbreviations used are: ROS, reactive oxygen species; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; NGF, nerve growth factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAPK, mitogen-activated protein kinase; NO, nitric oxide; DMEM, Dulbecco’s modified Eagle’s medium; DCFDA, 2′,7′-dichlorofluorescein diacetate; DCF, dichlorofluorescein; NAC, N-acetylcysteine; DPI, diphenylene iodonium; wt, wild-type; NBT, nitro blue tetrazolium; EMSA, electrophoretic mobility shift assay; PTPros(a), protein-tyrosine phosphatase(s); PAGE, polyacrylamide gel electrophoresis.
Neuronal Differentiation Requires Rac1-regulated ROS

RESULTS AND DISCUSSION

We first examined whether ROS production is detectable in PC12 cells upon NGF treatment. To detect ROS production, PC12 cells were preincubated with the peroxide-sensitive fluorophore DCF prior to NGF treatment. NGF stimulated ROS production in a dose-dependent manner, and the amount of ROS rapidly increased to its maximum level 10 min after NGF addition and thereafter decreased toward its basal level (Fig. 1A). By using confocal microscopy, we also confirmed that NGF induced an increase in DCF fluorescence in cells loaded with the fluorophore, DCF (data not shown). It was essential that PC12 cells were serum-starved before the NGF addition to avoid the serum effect on ROS production. We tested whether a chemical antioxidant scavenger can abolish the NGF-induced ROS. When cells were treated with NAC, NGF-stimulated DCF fluorescence was significantly reduced (83.9 ± 4% inhibition at 20 mM of NAC; the formula is in the legend to Fig. 1B).

In Situ Detection of Superoxide Production—PC12 cells were transfected with pcDNA-Rac1N17 or empty vector pcDNA and cultured in growth medium for 30 h, and NGF was added for 30 h. Cells were serum-starved in the presence of NGF for 12 h and with the medium containing NBT (5 mg/ml, Roche Molecular Biochemicals) and NGF for 30 min. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were incubated with anti-Rac1 antibody, reacted with fluorescein isothiocyanate-conjugated anti-mouse IgG, and labeled with Klenow fragments and [32P]dCTP. DNA-protein complexes were analyzed on a 4% polyacrylamide gel.

Detection of Catalase Effect—Cells were transfected with either P53CAT carrying human catalase or a control vector, P53. 36 h after transfection, cells were treated with NGF for 2 days, and the neurite outgrowth was determined. Values represent mean ± S.E. (n = 3). Catalase effect significantly reduced neurite outgrowth (35% inhibition at 200 units/ml catalase).

EMSA Assay—Preparation of nuclear extracts and the binding reaction were performed as described (17). The AP-1 binding sequence AGCTTGGATGACTCAG was annealed by the TCGACTGAGTCATCAA.
phagocytic cells, synthesis of ROS in response to agonists was catalyzed by the NADPH oxidase (3). Several growth factors and Ras-mediated mitogenic signals also activate the production of superoxide by the NADPH oxidase-like enzyme in fibroblast cells (10–12, 18, 19). To determine whether an enzyme functionally similar to the NADPH oxidase is involved in NGF-induced ROS generation in PC12 cells, we treated PC12 cells with DPI, a specific inhibitor for flavoprotein that is a constituent of the NADPH oxidase complex. DPI addition abolished the rise in DCF fluorescence by NGF treatment (87.1 ± 2% inhibition at 20 μM of DPI; Fig. 1B).

We next assessed the effect of NAC and DPI on NGF-induced differentiation of PC12 cells. The increasing amount of NAC exerted an inhibitory effect on neurite outgrowth, which is a major hallmark of the differentiation phenotype (Fig. 1C). This was also observed previously (20). Likewise, DPI treatment blocked this morphological differentiation induced by NGF (Fig. 1C). The cell viability was not affected by either NAC or DPI in the range of concentration used, as determined by trypan blue exclusion. These data suggest that ROS generated by NGF is required for NGF-induced neuronal differentiation and that activation of the phagocytic NADPH oxidase-like enzyme system mediates NGF-induced ROS synthesis. Rotenone (50 μM), a mitochondrial oxidase inhibitor, had no appreciable effect on NGF-induced ROS production, which indicates that the mitochondrial oxidase is not involved in this event (data not shown).

Because the NADPH oxidase is regulated by Rac1 in phagocytic cells and similarly super oxide generation appears to be modulated by Rac1 in fibroblast cells (10–12, 18, 19), we investigated the role of Rac1 in NGF-induced ROS generation in PC12 cells. The level of NGF-induced ROS in wt Rac1-transfected cells was slightly higher than that observed in control cells (Fig. 2A). Rac1N17, a dominant negative Rac1 significantly decreased the level of NGF-induced ROS (Fig. 2A). The constitutively active Rac1V12 markedly elevated DCF fluorescence without NGF treatment (Fig. 2A). The increase in the ROS level was reduced when Rac1V12-transfected cells were treated with 20 μM DPI (DPI-treated, DCF fluorescence 7.0 ± 2.6; DPI-untreated, DCF fluorescence 27.5 ± 8.0, n = 3). Immunoblotting analysis confirmed that the similar amount of wt Rac1, Rac1N17, and Rac1V12 proteins were expressed. These data suggest that induction of ROS generation by NGF was mediated by Rac1 and that Rac1 could regulate the DPI-sensitive oxidase system similar to the NADPH oxidase in phagocytes. Immunohistochemical study demonstrated that NGF-induced neurite outgrowth was abrogated by transient expression of the dominant negative Rac1N17 (39 ± 5% neurite extending cells, n = 3), whereas vector control had no suppressive effect on the morphological differentiation (93 ± 2% neurite extending cells, n = 3). Rac1N17 expressing cells with the
undifferentiated phenotype reduced ROS production, as assessed by NBT reduction assay, which has been used to detect superoxide production by the NADPH oxidase (21) (Fig. 2B). Normally differentiated cells lacking Rac1N17 expression exhibited strong NBT staining (Fig. 2B). The correlation between Rac1N17 expression and inhibition of both ROS production and neurite outgrowth strongly suggests that NGF-induced ROS-dependent neuronal differentiation is mediated by Rac1.

One of the well characterized intracellular signaling events induced by NGF is NGF receptor-mediated tyrosine phosphorylation (14, 15), and ROS is known to modulate the redox state of tyrosine-phosphorylated proteins (22). We examined the effect of antioxidants on the level of phosphotyrosine-containing proteins. 6–24 cells were analyzed because the level of tyrosine-phosphorylated proteins in parental PC12 cells was not high enough to be detected by the antibody used. Tyrosine phosphorylation of cellular proteins was increased upon NGF stimulation, and some of them could be downstream targets of TrkA (Fig. 3). Consistent with the reduction in ROS production and neurite outgrowth, NGF-stimulated tyrosine phosphorylation of these proteins was inhibited by NAC or DPI treatment. The data indicate that NGF-induced ROS may affect the steady state of tyrosine phosphorylation of various cellular proteins. DPI inhibition of phosphorylation indicates that the phagocytic NADPH oxidase-like enzyme participates in the regulation of tyrosine phosphorylation. Because AP-1 or NF-xB is known to be activated by ROS in some biological systems, we examined whether NGF induces ROS-mediated activation of these transcription factors. Gel shift assay demonstrated that nuclear extracts from NGF-treated cells activated the AP-1 activity and that NAC suppressed this activation (Fig. 4). The inhibitory effect on the AP-1 activity was also observed with DPI treatment (data not shown). This indicates the mediating role of ROS in the NGF-dependent activation of AP-1. No stimulation of the NF-xB activity by NGF was detected until at least 24 h after NGF treatment, suggesting that NF-xB is not involved in NGF signaling (data not shown).

To analyze the nature of ROS mediating NGF action, we transfected PC12 cells with a catalase expression plasmid. Catalase scavenges H2O2 by catalyzing the dismutation of H2O2 to H2O and O2. Catalase prevented NGF-induced neurite outgrowth (80% decrease), abolished NGF-induced DCF oxidation (Fig. 5, A and B), and inhibited NGF-dependent tyrosine phosphorylation (Fig. 5C). We observed that whereas NGF rapidly increased tyrosine phosphorylation of TrkA, the overexpression of exogenous catalase in the cells suppressed this phosphorylation. Therefore, a 140-kDa prominent tyrosine-phosphorylated band detected in the experiments (Figs. 3 and 5C) is most likely TrkA. The amount of transduced catalase into the cells was 10 times that of the endogenous enzyme (data not shown). NGF action was inhibited when a recombinant adenovirus carrying human catalase was infected into the cells was 10 times that of the endogeneous enzyme (data not shown). NGF action was inhibited when a recombinant adenovirus carrying human catalase was infected into the cells and neurite outgrowth strongly suggests that NGF-induced ROS plays a critical role in the NGF signaling pathway. Downstream of Ras, involves Rac1-mediated generation of ROS leading to AP-1 activation and the Ras/Raf/MEK/MAPK-dependent pathway. Our observation suggests that the NGF-induced H2O2 may be responsible for tyrosine phosphorylation of proteins including the TrkA receptor. Likewise, tyrosine phosphorylation of MAPK was blocked by inhibition of the PDGF-induced increase in H2O2 (10) and tyrosine phosphorylation of EGF receptor was blocked by depletion of EGF-induced H2O2 (25). Because H2O2 inactivates certain types of protein-tyrosine phosphatases (PTPases) in vitro (26), the inactivation of PTPase might aid the maintenance of the maximal tyrosine phosphorylation in the cells stimulated by the growth factors including NGF.

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