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 Rac1N17 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.

Reactive oxygen species (ROS) that cause oxidative stress have generally been viewed as cytotoxic depending on the dose (1, 2). ROS are responsible for the host defense mechanism in neutrophils (3) and possess carcinogenic potential associated with tumor promotion (4, 5). Recent studies, however, indicate that small nontoxic amounts of ROS may play a normal role as a second messenger in the various signaling pathways (1).

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 action 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 cytostasis 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 (DCFH-DA, 5 μM/mL) for 10 min at 37 °C and then the indicated amounts of NGF (Upstate Biologicals) were added. The DCF fluorescence intensity was measured by a CytoFluor 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 pretreated 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 orthovandate, 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-DI.

DCFH-DA, 2,7′-dichlorofluorescein diacetate; DCF, dichlorofluorescein; NAC, N-acetylcysteine; DPI, diphenylene iodonium; wt, wild-type; NBT, nitro blue tetrazolium; EMSA, electrophoretic mobility shift assay; PTPase(s), protein-tyrosine phosphatase(s); PAGE, polyacrylamide gel electrophoresis.
with LipofectAMINE and LipofectAMINE PLUS solution (Life Technologies). Cells were serum-starved 36 h after transfection and loaded with DCFHDA for 10 min before assessing the DCF fluorescence level. The expression levels of transfected wt Rac1 and Rac1 mutant proteins were detected by immunoblotting with monoclonal anti-Rac1 antibody (Upstate Biologials).

**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 observed by confocal fluorescence microscopy.

**EMSA Assay—**Preparation of nuclear extracts and the binding reaction were performed as described (17). The AP-1 binding sequence AGCTTTGATGACTCAG was annealed by the TCGACTGAGTCATCAA

**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

![Image](http://www.jbc.org/)

**Fig. 1.** NGF stimulates production of ROS, and its inhibition by NAC and DPI blocks NGF-induced neurite outgrowth. A, NGF-stimulated production of ROS. PC12 cells were preloaded with DCFHDA and treated with NGF for 10 min (a) or with NGF (50 ng/ml) for different time intervals (b). DCF fluorescence intensity was measured as described. The relative DCF fluorescence intensity (arbitrary) shown is from duplicate experiments. B, inhibition of NGF-induced ROS production by NAC and DPI. Serum-starved PC12 cells were pretreated with NAC (20 mM) and DPI (20 μM). Cells were loaded with DCFHDA and treated with NGF (50 ng/ml). DCF fluorescence intensity was measured. Values represent mean ± S.E. (n = 3). The formula of percent inhibition is as follows: (DCF (none) – (DCF + DPI or NAC) – (DCF – (none))) × 100; n = 3. C, inhibition of NGF-induced neurite outgrowth by NAC. After pretreatment with NAC, cells were treated with NGF (50 ng/ml) in the presence of the inhibitors. At day 3, photomicrographs were taken. Only cells with neurites longer than two cell diameters were scored as positive.

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**Fig. 2.** Rac1 regulates NGF-induced ROS production and differentiation. A, effects of Rac1 on NGF-stimulated DCF fluorescence. PC12 cells were transfected with wt Rac1 DNA, Rac1N17 DNA, Rac1V12 DNA, and empty pcDNA vector, and DCF fluorescence was determined. Values represent mean ± S.E. (n = 3). For protein expression analysis, proteins were subjected to SDS-PAGE (12%) and immunoblotted with anti-Rac1 antibody. The lanes for the empty vector show the level of endogenous wt Rac1. B, effect of Rac1N17 on morphology and ROS production. PC12 cells were transfected with Rac1N17 DNA, treated with NGF, and incubated with NBT as described. Cells were immunostained with anti-Rac1 antibody. Arrows indicate the cells expressing Rac1N17. Note the low level of NBT stain and that these cells show undifferentiated morphology. Arrowheads indicate differentiated cells with neurites that lack Rac1N17 expression and exhibit the high level of NBT stain.

![Image](http://www.jbc.org/)

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 extending cells were scored. Alternatively, 36 h after transfection, cells were deprived of serum for 12 h and loaded with DCFHDA and NGF prior to determining the DCF fluorescence intensity. Lysates were immunoblotted with anti-catalase antibody (Calbiochem). 6–24 cells were transfected with P53CAT or an empty vector, and tyrosine phosphorylation was analyzed by immunoblotting.
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 produc-
tion of superoxide by the NADPH oxidase-like enzyme in fibro-
blast 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 constit-
uent 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. 1 B).

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. 1 C). This
was also observed previously (20). Likewise, DPI treatment
blocked this morphological differentiation induced by NGF
(Fig. 1 C). 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 en-
zyme 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 phago-
cytic cells and similarly super oxide generation appears to be
modulated by Rac1 in fibroblast cells (10–12, 18, 19), we inves-
tigated the role of Rac1 in NGF-induced ROS generation in
PC12 cells. The level of NGF-induced ROS in wt Rac1-trans-
sected cells was slightly higher than that observed in control
cells (Fig. 2 A). Rac1N17, a dominant negative Rac1 signifi-
cantly decreased the level of NGF-induced ROS (Fig. 2 A). The
constitutively active Rac1V12 markedly elevated DCF fluores-
cence without NGF treatment (Fig. 2 A). 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). Immuno-
blotting 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-sensi-
tive oxidase system similar to the NADPH oxidase in phago-
cytes. 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 suppres-
sive effect on the morphological differentiation (93 ± 2% neu-
rite extending cells, n = 3). Rac1N17 expressing cells with the

![Fig. 3. Inhibition of NGF-induced tyrosine phosphorylation by NAC and DPI.](http://www.jbc.org/)

![Fig. 4. NAC inhibits NGF-induced stimulation of AP-1 activity.](http://www.jbc.org/)

![Fig. 5. Effects of overexpressed catalase on NGF signaling. A, NGF (50 ng/ml)-induced neurite outgrowth in PC12 cells transfected with PS3CAT (+) or an empty vector (-). Data represent mean ± S.E. (n = 3) and are obtained by scoring 50 cells as in the legend to Fig. 2. B, NGF-stimulated DCF fluorescence in PC12 cells transfected with PS3CAT (+) or an empty vector (-). Data are expressed as mean ± S.E. (n = 3). C, immunoblots of tyrosine-phosphorylated proteins from 6–24 cells and 6–24 cells overexpressing untreated catalase or catalase treated with NGF (50 ng/ml) for 10 min.)
Neuronal Differentiation Requires Rac1-regulated ROS

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-κB activity 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-κB activity by NGF was detected until at least 24 h after NGF treatment, suggesting that NF-κB 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 H$_2$O$_2$ by catalyzing the dismutation of H$_2$O$_2$ to H$_2$O and O$_2$. Catalase prevents 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 endogeneous enzyme (data not shown). NGF action was inhibited when a recombinant adenovirus carrying human catalase was infected into the cells (data not shown). These data implicate H$_2$O$_2$, a product of superoxide, as a potential mediator for NGF signaling.

Our data provide evidence that NGF stimulates generation of an oxygen radical, perhaps H$_2$O$_2$, which is required for NGF-induced neuronal differentiation. Considering that PDGF-stimulated cell DNA synthesis is mediated by H$_2$O$_2$ (10), ROS could serve as an essential regulator in differentiation and mitogenesis. Unlike the conventional view that an increased level of ROS is the possible cause of neurodegenerative diseases (23) and oxidative stress induces cell death (24), we identified that ROS at a narrow concentration range may function as a vital mediator for NGF receptor signaling. A recent study reported that the rise in NO, another radical following NGF stimulation, causes NGF-induced cytostasis, which is a critical event preceding neurite extension (16). ROS and NO could contribute in concert to the neurotrophic factor-promoted differentiation.

In response to NGF, Rac1 is most likely to activate the NADPH oxidase-like enzyme system similar to that in phagocytic cells. Presumably, 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 H$_2$O$_2$ 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 H$_2$O$_2$ (10) and tyrosine phosphorylation of EGF receptor was blocked by depletion of EGF-induced H$_2$O$_2$ (25). Because H$_2$O$_2$ 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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Nerve Growth Factor-induced Neuronal Differentiation Requires Generation of Rac1-regulated Reactive Oxygen Species

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