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Rapid suppression of free radical formation by nerve growth factor involves the mitogen-activated protein kinase pathway

(neurotrophin/reactive oxygen species/confocal microscopy/mitochondria/cell culture)

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ABSTRACT **Neurotrophins such as nerve growth factor (NGF) regulate neuronal survival during development and are neuroprotective in certain models of injury to both the peripheral and the central nervous system. Although many effects of neurotrophins involve long-term changes in gene expression, several recent reports have focused on rapid effects of neurotrophins that do not involve synthesis of new gene products. Because enhanced formation of reactive oxygen species (ROS) represents one consequence of many insults that produce neuronal death, we hypothesized that neurotrophins might influence neuronal function and survival through acute alterations in the production of ROS. Using an oxidation-sensitive compound, dihydrorhodamine, we measured ROS formation in a central nervous system-derived neuronal cell line (GT1-1 trk) and in superior cervical ganglion neurons, both of which express the transmembrane NGF receptor tyrosine kinase, trkA. There was enhanced production of ROS in both cell types in the absence of NGF that was rapidly inhibited by application of NGF; complete inhibition of ROS generation in GT1-1 trk cells occurred within 10 min. NGF suppression of ROS formation was prevented by PD 098059, a specific inhibitor of MEK (mitogen/extracellular receptor kinase, which phosphorylates mitogen-activated protein kinase). The observation that NGF acutely blocks ROS formation in neurons through activation of the mitogen-activated protein kinase pathway suggests a novel mechanism for rapid neurotrophin signaling, and has implications for understanding neuroprotective and other effects of neurotrophins.**

Neurotrophins, such as nerve growth factor (NGF), constitute a family of trophic factors that are critical for normal nervous system development as well as for the survival and maintenance of many populations of neurons (for review, see ref. 1). Upon binding to specific transmembrane tyrosine kinase (trk) receptors, neurotrophins activate several downstream intracellular pathways, including ras–mitogen-activated protein kinase (MAPK), PI3 kinase, and the phosphoprotein SNT, which result in long-term changes in gene expression (2–6). Recently, several groups have shown that neurotrophins also have acute effects on neuronal function and physiology (7–10). Neurotrophins have been reported to rapidly enhance synaptic transmission in both the peripheral and the central nervous system through activation of presynaptic (7–9) as well as possibly postsynaptic (10) neuronal trk receptors with a resulting increase in neurotransmitter release and synaptic strength. Neurotrophins, such as brain-derived neurotrophic factor, have also been reported to cause rapid changes in intracellular calcium in primary cultures of hippocampal neurons, an increase that depends on extracellular calcium (for review, see ref. 8). Since production of neuronal reactive oxygen species (ROS) is calcium-dependent in many systems (11, 12) and some effects of neurotrophins are rapid, we speculated that trk activation might lead to rapid alterations in the formation of ROS, such as superoxide anion, hydroxyl radical, and H_2O_2.

We investigated the effects of trk signaling on ROS formation in two cells types, GT1-1 trk cells and superior cervical ganglion (SCG) neurons. GT1-1 trk cells, an immortalized hypothalamic neuronal cell line (13) stably transfected with trkA (14), have many neuronal properties, including expression of specific neuronal structural proteins, synaptic vesicle proteins, gonadotropin releasing hormone, and functional γ-aminobutyric acid type A receptor complexes (13–15). In addition, they have spontaneous action potentials (15). Whereas parental GT1-1 cells do not express trkA or $p75^{NGFR}$ (14), GT1-1 trk cells exposed to NGF have rapid induction of trk phosphorylation, rapid and transient increases in mRNA for the immediate early genes c-*fos* and *NGFIA*, enhanced neuronal differentiation, and delayed cell death due to serum deprivation (14). We also chose to study NGF effects on ROS production in cultured SCG neurons. These neurons, which undergo programmed, apoptotic cell death 24–48 h after withdrawal of NGF *in vitro* (16), generate ROS during the first 1–4 h after NGF deprivation (17) and can be rescued from NGF-deprivation-induced degeneration by late re-addition of NGF. In SCG neurons, ROS may provide an early signal to mediate apoptosis (17). To determine whether neurotrophins can affect ROS production, we followed generation of ROS in both GT1-1 trk cells and in SCG neurons under different conditions in the presence or absence of NGF using confocal microscopy and an oxidation-sensitive fluorescent dye, dihydrorhodamine (DHR).

METHODS

Generation of Cell Lines. GT1-1 trk cells were generated by stably transfecting GT1-1 cells with a trkA expression vector (14); both GT1-1 and GT1-1 trk cells were cultured as described (14) in DMEM containing 5% horse serum and 5% fetal calf serum.

SCG Neuron Cultures. Neuronal cultures were prepared from the SCG of embryonic day 21 or postnatal day 0 rats as described (16). The neurons were plated on collagen-coated dishes and maintained at 37°C in a humidified atmosphere (5% CO_2 in air) for 6 days in the presence of NGF (50 ng/ml).

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Abbreviations: NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; SCG, superior cervical ganglion; DHR, dihydrorhodamine; MEK, MAPK/extracellular signal-regulated kinase kinase; trk, tyrosine receptor kinase; ROS, reactive oxygen species.

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Confocal Microscopy and Assessment of ROS Formation in GT1-1 and GT1-1 trk Cells. For imaging, suspensions of GT1-1 or GT1-1 trk cells were diluted and plated onto 35-mm culture dishes (Mat-Tek, Ashland, MA) possessing an oval cut-out sealed by a glass coverslip coated with poly-D-lysine: laminin (12). Cultures were analyzed when <60% confluent. The serum-containing culture medium was replaced with Hepes/bicarbonate-buffered salt solution, and cells were loaded with 10 μM DHR for 20 min at 37°C in a 5% CO_2 incubator. Oxidation of DHR to rhodamine 123 (E_x λ 488 nm, E_m $\lambda > 515$ nm) was followed at room temperature using a laser scanning confocal microscope (Noran Odyssey, Noran Instruments, Middleton, WI) with a ×60 water-immersion objective as previously described (12). Five fields of cells were obtained from each dish at each time point, and frame-averaged confocal images were digitized at 640 × 480 pixels using microcomputer-based image-analysis software (METAMORPH, Universal Imaging, Media, PA). For analysis of fluorescence intensity, regions for fluorescence quantitation included neuronal cytoplasm (excluding the nucleus); values reported here represent average pixel intensities within identified cells, with a range from 0 to 255. Data presented represent change in DHR fluorescence as a percentage of time 0 values. Control experiments confirmed that decreased fluorescence in the presence of NGF was not due to loss of rhodamine 123 from cells, or to insufficient DHR loading. A trkA–IgG fusion protein (18) was used in some experiments to determine whether it would block NGF actions on ROS production. Inhibitors of ROS-generating pathways (rotenone and meclofenamate) were added with DHR, resulting in a 30-min pretreatment. To determine the Ca^{2+} dependence of DHR oxidation, cells were exchanged into Hepes/bicarbonate-buffered salt solution without Ca^{2+} (normal Ca^{2+} concentration = 1.8 mM) during DHR loading, and were maintained in low Ca^{2+} for the duration of the experiment. Because of the length of the experiment, EGTA was omitted to avoid toxicity seen with complete removal of calcium.

Imaging of ROS in SCG Neurons. SCG neurons were evaluated after DHR loading ($t = 0$), and every hour thereafter, and were returned to the 37°C incubator between time points. For experiments in which NGF was re-applied to SCG cultures after a period of deprivation, it was necessary to remove the medium entirely (to dilute the anti-NGF antibody; ref. 19), and re-add NGF in solution containing a second aliquot of DHR (10 μM). Because of contaminating fluorescent rhodamine 123 in the DHR stock, fluorescence was increased slightly soon after this media exchange. To control for this, all control (+NGF) and deprivation (−NGF) cultures in each set of experiments underwent similar media removal and re-addition of DHR. For experiments using the MEK (MAPK/extracellular signal-regulated kinase kinase) inhibitor, PD 098059 (20, 21), the drug was included with the DHR loading to provide a 30-min pretreatment.

Western Blots for Phosphorylated MAPK. Cultures of GT1-1 trk cells (70% confluent) were incubated in serum-free medium for 3 h and treated with NGF for 10 min. Cells were then homogenized in Laemmli buffer and subjected to SDS/PAGE. After electrophoresis, proteins were transferred to poly(vinylidene difluoride) membranes (Millipore), then the blots were stained with a phospho-specific MAPK antibody (New England Biolabs) diluted 1:1000 as per the manufacturers instructions. Equal loading of samples was checked by stripping the blots and reprobing with an antibody that detects total MAPK (1:1,000; New England Biolabs).

RESULTS

ROS Formation by GT1-1 trk Cells Is Suppressed by Application of NGF. Using an oxidation-sensitive dye, DHR 123, and confocal fluorescence microscopy, we examined the formation of ROS in GT1-1 trk cells in the absence of NGF. Under baseline conditions (−NGF, no serum), we observed substantial formation of ROS in these cells (Fig. 1 *Upper*). The formation of ROS was markedly suppressed by the addition of 100 ng/ml NGF (Fig. 1 *Lower*). Inhibition of ROS by NGF was rapid, and production was already significantly less than that under control conditions by 10 min after NGF application (Fig. 2*A*). The NGF suppression of ROS production persisted through the 40-min observation period, to more than 1 h (data not shown). To verify that NGF suppression of ROS formation was due to an interaction with the NGF receptor, trkA, NGF was coapplied with trkA–IgG, a fusion protein containing the extracellular domain of TrkA and the Fc region of human IgG5, which competes for NGF with trkA receptors present on cells (18). TrkA–IgG eliminated the ability of NGF to suppress ROS formation (Fig. 2*A*). To further confirm that ROS

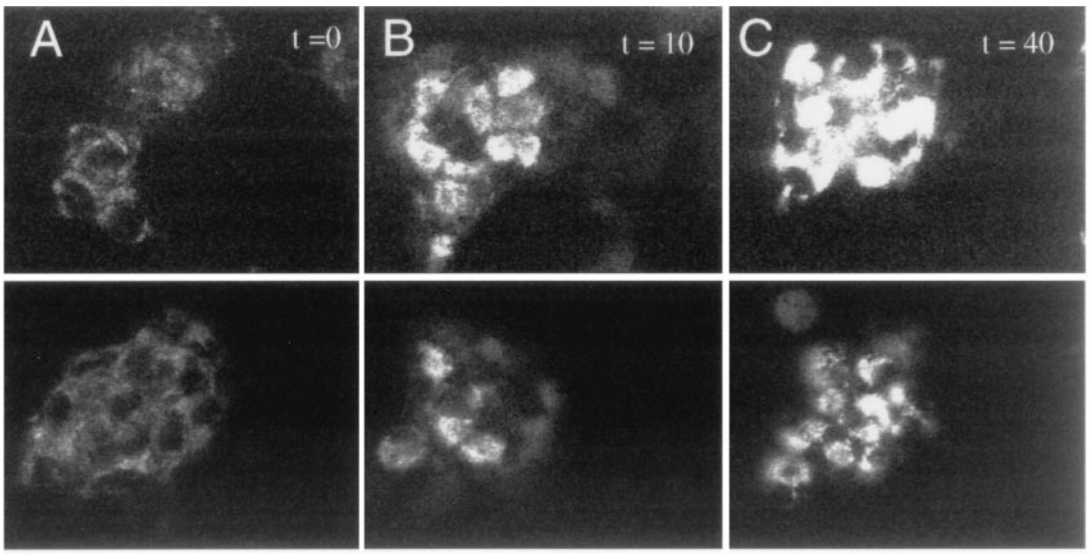

FIG. 1. Confocal photomicrographs of DHR fluorescence in GT1-1 trk cells in the absence (*Upper*) or presence (*Lower*) of NGF. Fluorescent images obtained immediately after loading (A, $t = 0$) show low basal fluorescence. Cells were then treated with vehicle (media; *Upper*) or NGF (100 ng/ml; *Lower*), and follow-up images were obtained at 10 min (B) or 40 min (C). A substantial increase in staining with rhodamine 123, the fluorescent oxidation product of DHR, can be seen in the absence of NGF but not in the presence of NGF.

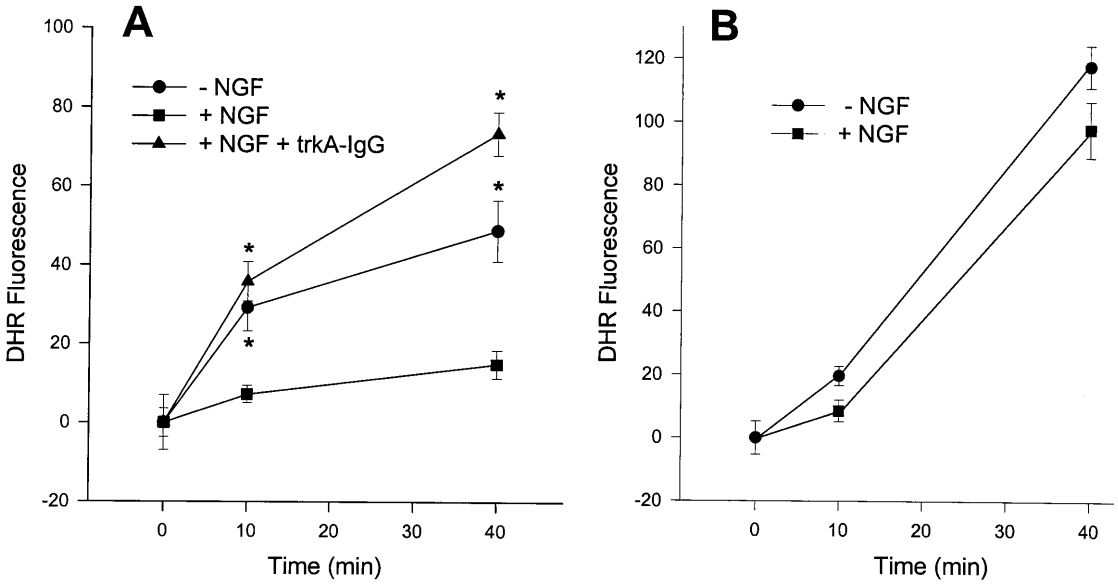

FIG. 2. NGF rapidly suppresses free radical formation in a trkA-dependent manner. Generation of ROS in the absence of NGF results in increased DHR fluorescence in GT1-1 trk cells (*A*), which possess functional trkA receptors, as well as in parental GT1-1 cells (*B*), which lack the trkA receptor. However, DHR oxidation in GT1-1 trk cells was suppressed within 10 min of adding NGF (*A*), and this suppression was blocked by trkA–IgG, which competes with native trkA receptors for NGF. In contrast, NGF failed to suppress ROS formation in parental GT1-1 cells that do not express trkA receptors (*B*). Values presented represent the change in DHR fluorescence as a percentage of time 0 values. Values are the mean ± SEM; n = 25–75 cells total from 5 fields imaged per condition per time point. Significance was determined by ANOVA, followed by Bonferroni test for multiple comparisons. *, $P < 0.05$.

suppression requires functional trkA, ROS formation was measured in the parental GT1-1 cells, which lack trkA receptors (14). GT1-1 cells demonstrate substantial basal ROS formation in serum-free medium that was insensitive to the addition of NGF (Fig. 2*B*). These results indicate that suppression of ROS by NGF is mediated through trkA, not through antioxidant properties of the NGF protein or solution.

Sites of ROS Formation in GT1-1 trk Cells. To identify the source(s) of ROS production observed in GT1-1 trk cells in the absence of NGF, we examined the effect of pharmacological inhibition of several ROS-generating pathways on DHR oxidation. Since GT1-1 trk cells have no demonstrable nitric oxide synthase protein or activity (unpublished results), nitric oxide cannot be the source of ROS production. However, rotenone, an inhibitor of mitochondrial electron transport at NADH dehydrogenase (complex I) in the mitochondrial electron transport chain, significantly reduced DHR oxidation. Meclofenamate, an effective inhibitor of arachidonic acid metabolism through cyclooxygenase, 5- and 12-lipoxygenase (S. Hewett, D. Choi, and L.L.D., unpublished results) also reduced ROS formation substantially. In addition to effects of

Table 1. DHR fluorescence in GT1-1 trk cells

Condition	10 min	40 min
+ NGF	8 ± 4	44 ± 5
− NGF	57 ± 5*	181 ± 9*
+ 100 μM Meclofenamate	30 ± 2	125 ± 6†
+ 20 μM Rotenone	22 ± 6†	94 ± 14†
NGF + 10 μM PD98059	19 ± 4	53 ± 4
NGF + 50 μM PD98059	54 ± 6*	152 ± 7*
NGF + 100 μM PD98059	68 ± 5*	179 ± 8*
No calcium		
+ NGF	35 ± 4	86 ± 8†
− NGF	30 ± 5	84 ± 7†

Free radical production in GT1-1 trk cells detected as increased fluorescence due to oxidation of DHR to rhodamine 123. Values represent the percent change in brightness from basal values obtained just after loading DHR 123. All drugs were added as a 30-min pretreatment by including the drug with the DHR loading solution. Solutions used for most experiments contained 1.8 mM calcium, whereas solutions for the low-calcium condition contained no added calcium but did not include a calcium chelator to drop the calcium concentration to 0. Values represent the mean ± SEM for n = 40–300 for each group.
*Versus +NGF condition. $P < 0.05$ by ANOVA followed by Student–Newman–Keuls.
†Versus −NGF condition. $P < 0.05$ by ANOVA followed by Student–Newman–Keuls.

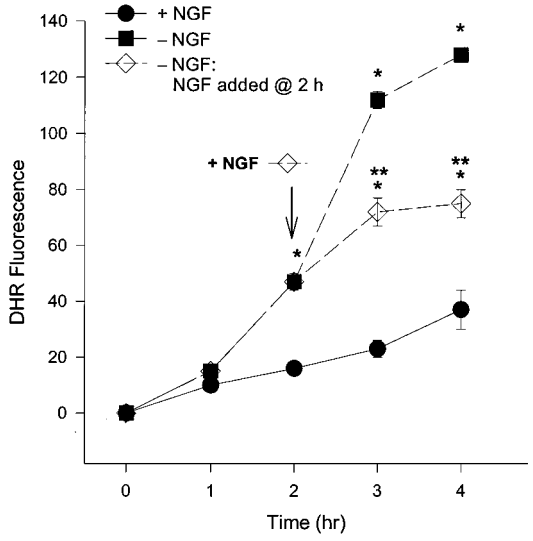

FIG. 3. NGF suppresses free radical formation induced by NGF deprivation in primary cultures of SCG neurons. SCG neurons were loaded with DHR, and NGF deprivation was initiated by removal of media containing NGF, plus addition of an anti-NGF antiserum. Increased fluorescence was observed between 1 and 2 h after removal of NGF, and the rate of fluorescence increase was greatest between 2 and 4 h after removal of NGF. This fluorescence increase was not observed if cultures were maintained in NGF. Reintroduction of NGF after 2 h attenuated the increase in fluorescence, suggesting that subsequent ROS formation was blocked by NGF. Values are the mean ± SEM (n = 12–36). *Versus +NGF condition. **Versus −NGF condition. $P < 0.05$, by ANOVA followed by Bonferroni test for multiple comparisons.

inhibitors of specific ROS-generating pathways, we also found that ongoing ROS formation in the absence of NGF was Ca^{2+}-dependent (Table 1) and showed near-complete inhibition in low-calcium medium. We were unable to determine whether the inhibition of ROS by NGF was Ca^{2+}-dependent, however, since removal of calcium from the medium blocked ROS generation independent of the presence of NGF (Table 1).

ROS Formation in SCG Neurons During NGF Deprivation. We then determined whether NGF could suppress ROS formation in primary cultures of SCG neurons. In SCG neurons deprived of NGF, we detected increased ROS formation 1–2 h after removal of NGF (plus addition of anti-NGF antibody; ref. 19), with a dramatic increase in ROS between 2–4 h (Fig. 3). Cultures maintained in NGF failed to exhibit enhanced ROS production during the same period. When NGF was added back to cultures 2 h after it was removed, subsequent ROS formation was significantly suppressed within the next hour (Fig. 3). Re-addition of NGF at 1 h (which precedes the major increase in ROS production) also rapidly suppressed ROS formation (data not shown).

The MAPK Pathway Is Necessary for NGF Attenuation of ROS Production. PD 098059, a specific inhibitor of MEK reported to have no effect on PI3 kinase, or other serine/threonine or tyrosine kinases (20, 21), blocks phosphorylation of MAPK in GT1-1 trk cells exposed to NGF (Fig. 4*A*). When GT1-1 trk cells were treated with PD 098059 (100 μM) in the presence of NGF, the inhibitor significantly reversed the ability of NGF to suppress ROS formation (Fig. 4*B*). PD 098059, previously reported to block MAPK activation in SCG neurons (21), also reversed ROS suppression by NGF in SCG neurons at a similar concentration (100 μM; Fig. 4*C*).

DISCUSSION

To our knowledge, this is the first report demonstrating rapid suppression of cellular ROS formation by a neurotrophin. The

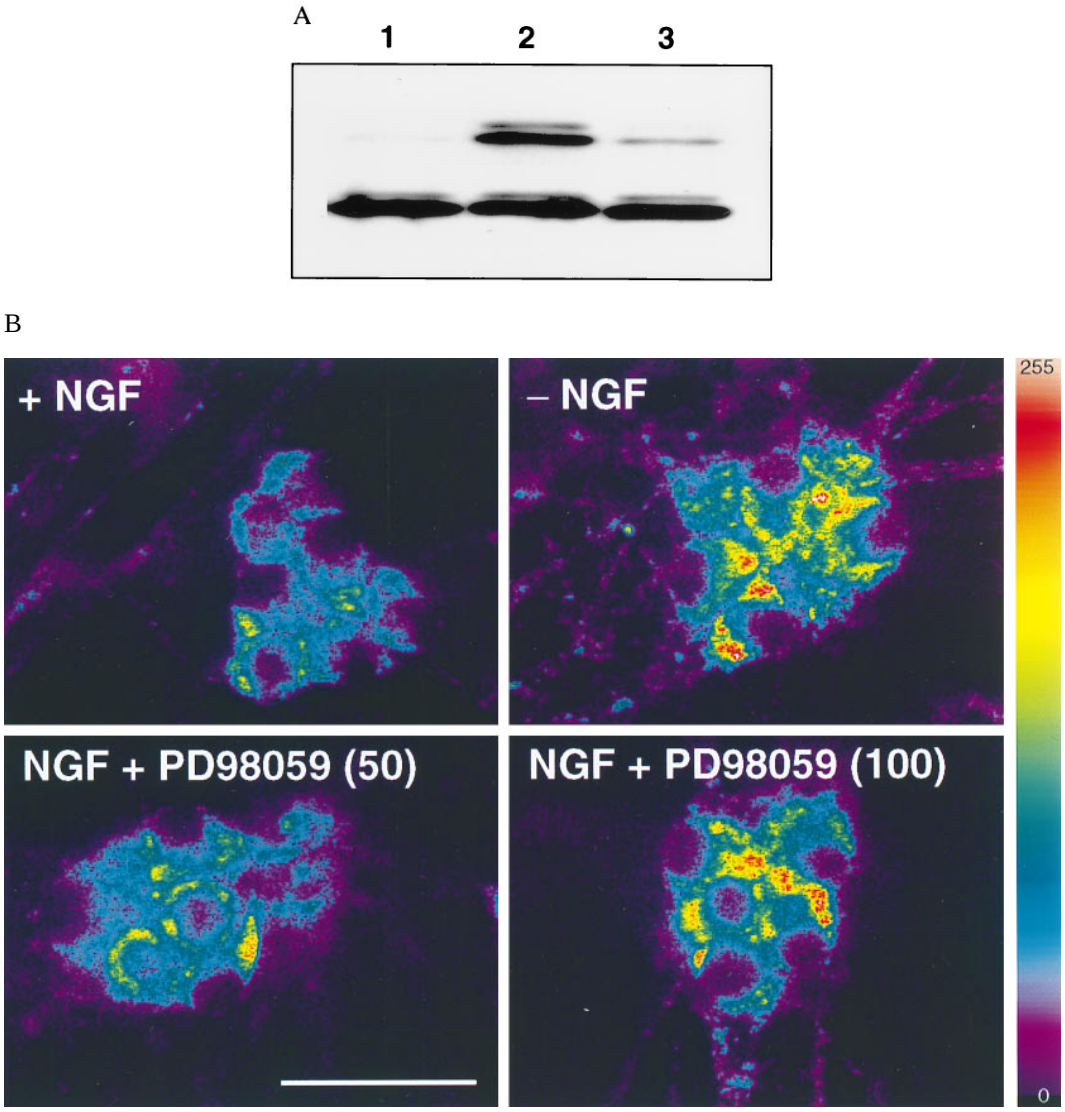

FIG. 4. The MEK inhibitor, PD 098059, blocks both NGF-mediated activation of MAPK and suppression of ROS formation. *(A)* MAPK activation in GT1-1 trk cells was determined by probing Western blots of cell lysates with a phospho-specific MAPK antibody (upper bands). The same blot was then stripped and reprobed with a control MAPK antibody that recognizes both phosphorylated and nonphosphorylated forms of ERK-1 and ERK-2 (lower bands). Lanes: 1, lysate from untreated GT1-1 trk cells (controls); 2, lysate from cells treated with 50 ng/ml NGF for 10 min; 3, lysate from cells pretreated for 30 min with 100 μM PD 098059, then with 50 ng/ml NGF for 10 min. (*B*) Fluorescence photomicrographs of SCG neurons treated with NGF and PD 098059. SCG neurons loaded with DHR, then deprived of NGF at $t = 0$, demonstrated a substantial increase in fluorescence 3 h after removal of NGF, whereas cells maintained in NGF showed little oxidation of the dye. PD 098059 (50 μM and 100 μM) coapplied with NGF reversed suppression of ROS by NGF in a dose-dependent fashion. Images are pseudocolor representations of fluorescence intensities, using arbitrary fluorescence intensity units on the linear color scale. (Bar = 50 μm.)

results are consistent with an expanding literature documenting that trk signaling leads not only to changes in gene expression, which occur over hours and days, but also to rapid alterations in neuronal function, which can occur over minutes (7–10). Previous *in vitro* and *in vivo* studies have shown that neurotrophins, including NGF, can change expression of certain antioxidant enzymes (22, 23) as well as attenuate the glutamate-induced production of peroxides (24). These described effects, however, occur over days. Our results suggest that, like rapid neurotrophin/trk-induced changes in synaptic activity, trk signaling can also acutely alter ROS formation. As the production of ROS can alter cellular function in a variety of ways (for review, see ref. 25), these findings may have important implications for understanding mechanisms leading to neuronal ROS production and its consequences in both normal and disease states.

We found that basal ROS production was decreased by inhibitors of both the mitochondrial electron transport chain and arachidonic acid metabolism, suggesting two possibilities: (*i*) there are at least two separate sites of ROS formation in GT1-1 trk cells—the mitochondrial electron transport chain and arachidonic acid metabolism, or (*ii*) mitochondria are the major site of ROS formation, but mitochondrial production of ROS is modulated by products of arachidonic acid metabolism. In addition, we noted that basal ROS production in GT1-1 trk cells was calcium-dependent. Because of the duration of the experiments in SCG neurons, we were unable to determine whether early ROS formation after NGF withdrawal was reduced in zero-Ca^{2+} solutions because prolonged removal of extracellular Ca^{2+} is neurotoxic.

Whereas trk activation leads to a complex set of secondary events, activation of the ras–MAPK pathway through trk signaling (26–28) plays a key role in many effects of trk receptor signaling, including changes in gene expression and neuronal differentiation in PC12 cells (6). We hypothesized that NGF-dependent suppression of ROS might act through activation of MAPK, or one of its downstream targets. Ras-dependent phosphorylation/activation of MAPK is carried out by MEK (6, 26–28). Our results, using the specific MEK inhibitor PD 098059 (20, 21), support the importance of MAPK activation after NGF stimulation of the trkA receptor to rapidly block ROS production in both an immortalized hypothalamic neuronal cell line as well as in primary SCG neurons. Rapid modulation of free radical formation by the MAPK pathway may be one means whereby neurotrophins signal within the cell, potentially acting through redox-sensitive proteins such as transcription factors (29), neurotransmitter transporters (30), channels (31), kinases (32), and even ras itself (33). Further, less reactive radical species, such as hydrogen peroxide, superoxide anion, and nitric oxide, can diffuse out of cells with relative ease (25, 34). One additional consequence of trk receptor activation, therefore, might be intercellular signaling by neurons using radicals as short-range signaling molecules, providing neurons with an additional means to communicate with neighboring cells, including astrocytes, other neurons, immune cells, and endothelial cells.

Early production of ROS has been documented in hypoxic-ischemic and traumatic brain injury (for review, see ref. 35). In many of the same acute central nervous system injuries, neurotrophins and neurotrophin receptor mRNAs are upregulated within hours after the insult (36). Enhanced expression of neurotrophins is likely due to injury-induced glutamate release because neurotrophin expression can be induced by direct application of glutamate receptor agonists *in vitro* and *in vivo* (36–41). In light of our observation that NGF suppresses neuronal ROS production, one might speculate that changes in neurotrophin and trk gene expression are an attempted compensatory brain response to ameliorate ongoing central nervous system injury by suppressing postinjury ROS production. We have recently reported marked protective effects of both exogenous NGF (42) and brain-derived neurotrophic factor (43) in a neonatal model of hypoxic-ischemic brain injury. It will be interesting to determine whether suppression of ROS production contributes to the observed neuroprotective effects of these neurotrophins.

In summary, we have observed acute suppression of neuronal ROS production by NGF, and determined that activation of the MAPK pathway through trk signaling appears to be necessary for this action of NGF. Although previous studies have shown that neurotrophins can induce long-term changes in free radical detoxifying enzymes (22–24), rapid alteration of ROS production by NGF represents a novel means whereby neurotrophins may signal within neurons, or possibly to other nearby cells. Identifying and understanding these acute effects will likely have important implications for understanding the role of neurotrophins in normal neuronal function, as well as for the consequences of trk receptor activation in neurological disease states.

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