Antioxidant *N*-Acetylcysteine Blocks Nerve Growth Factor–Induced H_2O_2/ERK Signaling in PC12 Cells

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ABSTRACT: We investigated whether H_2O_2, superoxide, and ERK participate in nerve growth factor (NGF)–induced signaling cascades and whether antioxidant *N*-acetylcysteine (NAC) regulates these NGF-induced responses. PC12 cells were cultured in medium containing NGF or vehicle with or without NAC pretreatment, and the intracellular H_2O_2 and superoxide levels and the amount of phosphorylated ERK were evaluated by flow cytometry and Western blotting, respectively. We found that NGF increased intracellular H_2O_2 concentration and activated ERK but failed to affect intracellular superoxide level. Moreover, NAC counteracted these NGF-induced responses. These findings demonstrate that NAC blocks the NGF-induced H_2O_2/ERK signaling in PC12 cells.

KEYWORDS: antioxidant; *N*-acetylcysteine; PC12 cells; ERK; NGF; hydrogen peroxide; reactive oxygen species

INTRODUCTION

The cellular redox reaction plays a pivotal role in a variety of normal physiological functions (e.g., normal cardiovascular function,[1] normal sperm function,[2] and nerve growth factor [NGF] signaling[3,4]) and pathological processes.[5,6] Recent findings point out that reactive oxygen species (ROS) mediate many effects of angio-

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Ann. N.Y. Acad. Sci. 1042: 325–337 (2005). © 2005 New York Academy of Sciences.
doi: 10.1196/annals.1338.056

tensin II, which are vital for normal cardiovascular function.[1] Other evidence also indicates that the spermatozoa produce ROS, which promote sperm capacitation.[2] Moreover, ROS mediate NGF-induced neuronal differentiation[3] and phosphorylation of cAMP-responsive element-binding protein (CREB).[4] In contrast, overproduction of ROS leads to vascular injury associated with angiotensin II–dependent hypertension.[5] Accumulating evidence also suggests that oxidative stress contributes significantly to the development of neurodegenerative diseases.[6–8]

Hydrogen peroxide and superoxide are two common ROS that are generated in the cellular redox reaction and play an essential role in various signaling pathways, regulatory processes, or pathophysiological conditions.[9–12] Application of platelet-derived growth factor (PDGF) to vascular smooth muscle cells (VSMCs) induces activation of tyrosine phosphorylase and mitogen-activated protein kinase (MAPK), DNA production, and a brief increase of intracellular hydrogen peroxide levels.[9] Inhibition of hydrogen peroxide production blocks the PDGF-induced responses in the VSMCs mentioned above, suggesting that hydrogen peroxide mediates these PDGF effects on VSMCs.[9] Evidence indicates that hydrogen peroxide controls neuronal plasticity by altering the action of some special calcium-dependent phosphatases[12] and acts as a key second messenger to mediate the effects of metabolic oxidative stress.[11] Application of angiotensin II leads to elevated mean arterial blood pressure and increased production of superoxide anion in rats, which are decreased and counteracted by treatment of liposome-entrapped superoxide dismutase (SOD), suggesting that superoxide mediates, at least in part, the angiotensin II–induced hypertension in rats.[10]

NGF exerts a variety of effects on sensory neurons, sympathetic neurons, and pheochromocytoma (PC12) cells, including cell survival, neurite outgrowth, and cell differentiation, by binding to NGF receptors and activation of NGF signaling pathways.[13–16] Recent evidence indicates that NGF results in a momentary increase of intracellular ROS in PC12 cells and that antioxidant *N*-acetylcysteine (NAC) blocks this NGF-induced elevation of intracellular ROS.[3] Catalase counteracts the production of intracellular ROS, neurite growth, and activation of tyrosine phosphorylase induced by NGF, and therefore hydrogen peroxide is suggested to mediate these NGF-induced responses.[3] Nonetheless, the role of ROS in NGF-induced signaling and the downstream signaling cascades of ROS induced by NGF in PC12 cells remain poorly understood.

In this study, we tested the hypotheses that hydrogen peroxide, superoxide, and ERK activation play an important role in NGF-induced signaling in PC12 cells and that antioxidant NAC counteracts these NGF-induced responses in PC12 cells. Our results indicated that NGF had no effect on the intracellular concentration of superoxide in PC12 cells. Nonetheless, NGF increased the intracellular hydrogen peroxide level and the phosphorylation of ERK in PC12 cells, and these NGF-induced responses were blocked by NAC pretreatment. Moreover, a selective MAPK inhibitor, PD098059, suppressed phosphorylation of ERK. Taken together, these findings demonstrate that the production of hydrogen peroxide and activation of ERK are two important steps of NGF-induced MAPK signaling in PC12 cells and strongly suggest that hydrogen peroxide mediates the NGF-induced MAPK/ERK signaling.

MATERIALS AND METHODS

Cell Culture and Chemicals

In a humidified incubator with continuous aeration of 5% CO_2 and 95% air at 37°C, PC12 cells were incubated and maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, Logan, UT) containing penicillin G (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL), L-glutamine (2 mM), nonessential amino acids (1 mM), fetal bovine serum (FBS; 5%), and horse serum (10%). At the beginning of all experiments, unless otherwise stated, PC12 cells were plated onto poly-L-lysine–coated tissue culture dishes (10^6 cells/100-mm plate) including a low-serum DMEM medium with 1% FBS; the medium was replenished every 48 h.

Measurement of Intracellular Hydrogen Peroxide and Superoxide

For the measurement of hydrogen peroxide, following starvation in low-serum DMEM with 1% FBS for 24 h, PC12 cells were treated with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) for 20 min, and then NGF (100 ng/mL) (catalog no. 556-NG-100; R & D Systems, Minneapolis, MN) or vehicle was added to PC12 cells, which were collected 1, 3, 5, 10, and 20 min after NGF or vehicle application and then trypsinized for immediate detection of intracellular hydrogen peroxide. The dichlorodihydrofluorescein (DCFH), the deacetylated product of DCFH-DA by intracellular esterases, reacts with H_2O_2 to form dichlorofluorescein (DCF), which is an oxidized fluorescent compound. The amount of intracellular hydrogen peroxide can be quantified by detection of DCF using a flow cytometer with excitation and emission wavelengths set at 488 nm and 525–550 nm (FL1-H), respectively.[17]

In a separate experiment, we investigated whether antioxidant NAC has any effect on the NGF-induced elevation of intracellular hydrogen peroxide in PC12 cells because NGF treatment did increase the production of intracellular hydrogen peroxide in PC12 cells in the preceding experiment. In addition to the control group and the NGF treatment group, we added an additional group with NAC (5 mM) pretreatment (Sigma Chemical Co., St. Louis, MO) 4 h prior to addition of NGF (100 ng/mL). To determine whether NAC can clear the hydrogen peroxide entering PC12 cells, we also administered NAC to PC12 cells 4 h before addition of H_2O_2 (200 μM) (Merck & Co., Whitehouse Station, NJ) to PC12 cells besides the control group and the H_2O_2 treatment group. The intracellular concentration of hydrogen peroxide in PC12 cells was measured 1 min after NGF or H_2O_2 treatment by the same procedures described earlier.

For detection of superoxide, following starvation in low-serum DMEM with 1% FBS for 24 h, 5 μM dihydroethidium (HEt) was added to PC12 cells 5 min before addition of NGF (100 ng/mL) or vehicle. PC12 cells were collected 1, 3, 5, 10, and 20 min after NGF or vehicle treatment and then trypsinized for immediate evaluation of intracellular superoxide by using a flow cytometer. When HEt enters the cells, it will react with superoxide to form ethidium, which will be incorporated into the nuclear DNA[18] and can be detected by a flow cytometer with excitation and emission wavelengths set at 488 and 637 nm (FL2-H), respectively.

Western Blot Analysis

PC12 cells were incubated in a low-serum DMEM with 1% FBS for 24 h, and NGF (100 ng/mL) was added to the medium for 5, 10, 20, 40, 60, 120, and 180 min; PC12 cells without NGF treatment served as the negative control group. PC12 cells were scraped, washed, and lysed at a density of 10^6 cells/50 μL of lysis buffer (25 mM HEPES, 5 mM EDTA, 0.1 mM sodium deoxycholate, 1.5% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.5 M NaCl)[19] (Merck & Co., Inc.), including a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). PC12 cells were then incubated on ice for 20 min. Following centrifugation of the lysates at 15,000 × *g* and 4°C for 15 min, the amount of proteins in the supernatant was quantified using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA). After addition of sample buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 140 mM β-mercaptoethanol, 0.002% bromophenol blue), each lysate was boiled for 5 min and loaded for electrophoresis in an SDS–polyacrylamide gel (30 μg of protein/lane). Proteins on the gel were electrotransferred onto polyvinylidene difluoride (PVDF) membranes, and the PVDF membranes were then incubated with anti-ERK2 antibody (1:2000) (catalog no. sc-154; Santa Cruz Biotechnology, Santa Cruz, CA) or anti–phospho-ERK1/2 antibody (1:2000) (catalog no. 9101S; New England Biolabs, Beverly, MA). After several rinses, the PVDF membranes were incubated with appropriate horseradish peroxidase–conjugated secondary antibodies (1:10,000 dilution) and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ). The amount of total ERK1/2 (t-ERK1/2) or phosphorylated ERK1/2 (p-ERK1/2) was quantified by a densitometer (Gel Doc 2000; Bio-Rad Laboratories). For the relative ERK activity, the p-ERK1/2 over the t-ERK1/2 in the negative control group was calculated, set to 1.00, and used as the unit to express the ratio of p-ERK1/2 over the t-ERK1/2 for the remaining groups.

In an additional experiment, we examined whether antioxidant NAC blocks the NGF effect on the activation of ERK in PC12 cells, because NGF treatment activated ERK in PC12 cells in the previous experiment. In addition to the negative control group and the NGF treatment groups with or without dimethyl sulfoxide (DMSO) (Sigma Chemical Co.), we added additional groups with pretreatment of PC12 cells with different doses of NAC (5, 10, or 20 mM) 4 h prior to addition of NGF to PC12 cells. To determine whether ERK activation is a downstream event of MAPK in NGF-treated PC12 cells, we also applied different doses of a specific MAPK inhibitor PD098059 (10, 20, or 30 μM in DMSO solution) or DMSO 1 h prior to addition of NGF to PC12 cells. PC12 cells were collected 5 min after NGF treatment, and the amounts of p-ERK1/2 and t-ERK1/2 were measured by the same procedures described previously; the relative ERK activity was calculated and expressed in the same way described earlier.

RESULTS

Application of NGF (100 ng/mL) caused an increase of intracellular hydrogen peroxide but not superoxide levels in PC12 cells, and antioxidant NAC counteracted the NGF effect on the production of intracellular hydrogen peroxide. NGF treatment led

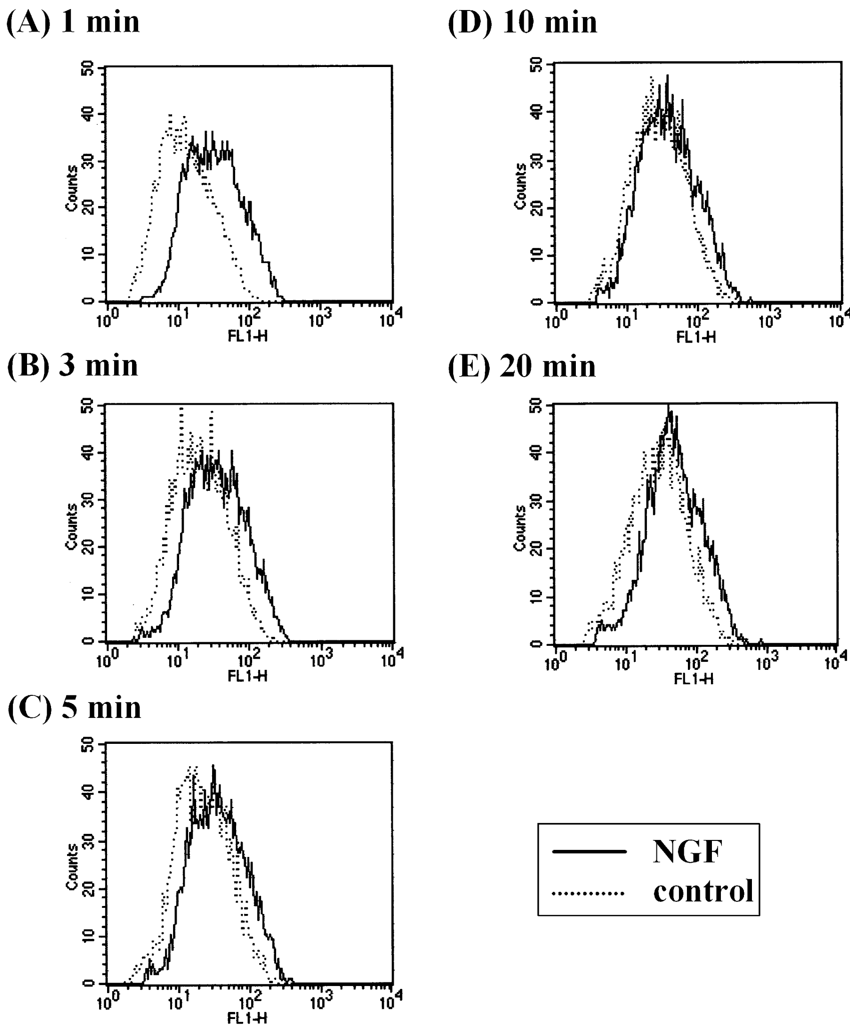


FIGURE 1. NGF induces generation of intracellular hydrogen peroxide in PC12 cells. PC12 cells were treated with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 min before addition of NGF (100 ng/mL) or vehicle. Intracellular level of hydrogen peroxide was measured by detection of dichlorofluorescein (DCF) in the FL1-H level by flow cytometry at (**A**) 1, (**B**) 3, (**C**) 5, (**D**) 10, and (**E**) 20 min after NGF or vehicle application. NGF caused a 3-, 1.8-, and 1.4-fold increase in intracellular hydrogen peroxide levels 1, 3, and 5 min, respectively, after application. Concentration of intracellular hydrogen peroxide returned to the control level 10 min after NGF application.

to an elevation of intracellular hydrogen peroxide measured by flow cytometry 1, 3, and 5 min after treatment when compared with the control group (FIG. 1A–C). The intracellular concentration of hydrogen peroxide induced by NGF reached the highest level 1 min after application (3 times the control value, FIG. 1A), decreased gradually between 3 min (1.8 times the control value, FIG. 1B) and 5 min (1.4 times the control value, FIG. 1C) after NGF administration, and returned to the control level 10 min after treatment (FIG. 1D). NAC pretreatment (5 mM) counteracted the NGF-induced twofold rise of intracellular hydrogen peroxide 1 min after NGF treatment in a separate experiment (FIG. 2A). Application of hydrogen peroxide (200 μM) to PC12 cells

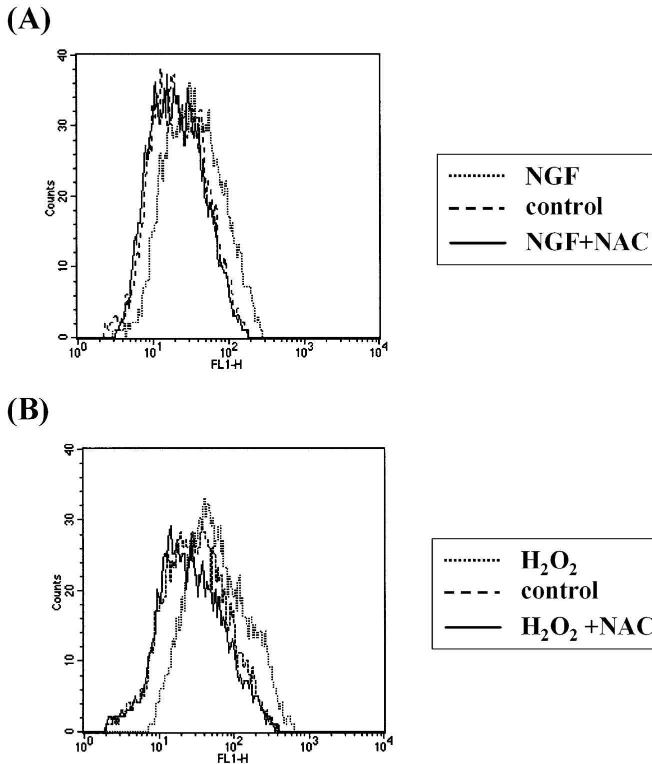


FIGURE 2. Antioxidant *N*-acetylcysteine (NAC) counteracts the (**A**) NGF-induced and (**B**) H_2O_2-induced production of hydrogen peroxide in PC12 cells. PC12 cells were pretreated with 5 mM NAC 4 h before NGF or H_2O_2 application. We applied 10 μM 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to PC12 cells 20 min before NGF (100 ng/mL), H_2O_2 (200 μM), or vehicle was added to the culture medium. Amount of dichlorofluorescein (DCF) in the FL1-H level that reflected the intracellular level of hydrogen peroxide was measured by flow cytometry at 1 min after NGF, H_2O_2, or vehicle application. (**A**) NGF increased the intracellular level of hydrogen peroxide in PC12 cells, and NAC pretreatment abolished this NGF effect. (**B**) Hydrogen peroxide caused an increase in intracellular hydrogen peroxide level in PC12 cells, and NAC pretreatment counteracted this hydrogen peroxide effect.

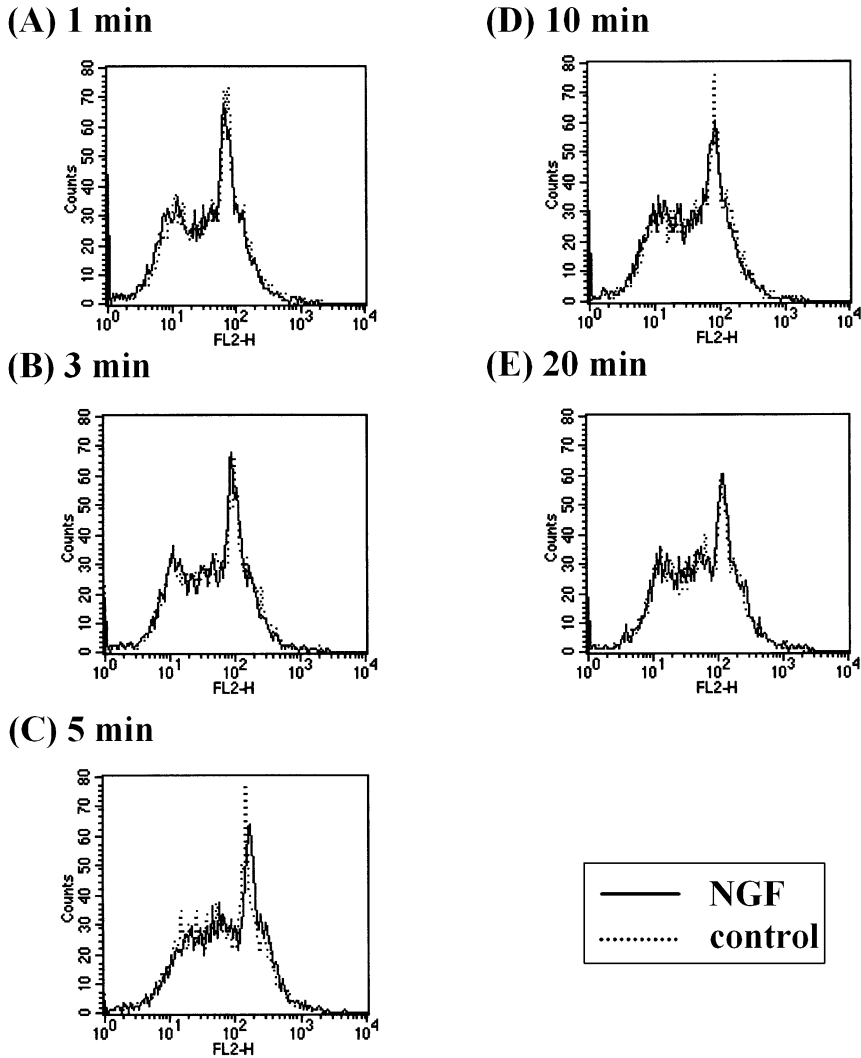


FIGURE 3. NGF has no effect on the generation of intracellular superoxide in PC12 cells. PC12 cells were treated with 5 μM dihydroethidium (HEt) for 5 min before addition of NGF (100 ng/mL) or vehicle. The intracellular level of superoxide was measured by detection of ethidium in the FL2-H level by flow cytometry at (**A**) 1, (**B**) 3, (**C**) 5, (**D**) 10, and (**E**) 20 min after NGF or vehicle application. Our data showed that NGF failed to affect the production of superoxide at all time points examined.

resulted in a 4.2-fold increase of intracellular hydrogen peroxide (FIG. 2B), and NAC pretreatment prevented this hydrogen peroxide effect (FIG. 2B). In contrast, NGF application failed to affect the level of intracellular superoxide in PC12 cells when compared with the control group at all time points examined (FIG. 3).

Western blot data showed that application of NGF (100 ng/mL) to PC12 cells induced a 2- to 8.7-fold increase in the relative ERK activity (p-ERK1/2 over t-ERK1/2) from 5–180 min after treatment (FIG. 4). The relative ERK activity reached 8.7 times the control value at 5 min after NGF application, dropped to ~ 4–5 times the control value 10–40 min after NGF treatment, and stayed at around 2 times the control value 1–3 h after treatment (FIG. 4B).

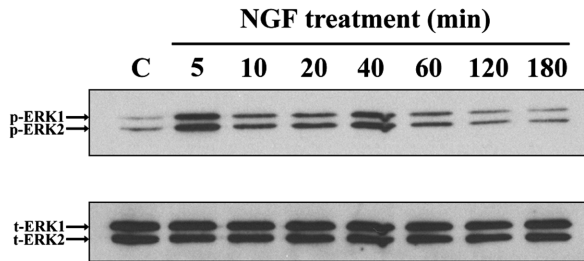
Antioxidant NAC or a selective MAPK inhibitor PD098059 pretreatment blocked the NGF-induced ERK activation in PC12 cells. NGF treatment alone (lane 2 of FIG. 5) caused a significant increase of ERK activation in PC12 cells when compared with the negative control group without NGF treatment (lane 1 of FIG. 5). Pretreatment of PC12 cells with antioxidant NAC 4 h before NGF application suppressed the NGF-induced activation of ERK at all three doses (5, 10, and 20 mM for lanes 7, 8, and 9 of FIG. 5, respectively) when compared with the positive control groups (lanes 2 and 3 of FIG. 5: lane 2, NGF only; lane 3, NGF plus DMSO) 5 min after NGF application. NGF plus DMSO (the solvent for PD098059, lane 3 of FIG. 5) caused a slight increase of ERK activity in PC12 cells when compared with the group receiving NGF alone (lane 2 of FIG. 5). Pretreatment of PC12 cells with a selective MAPK inhibitor PD098059 1 h prior to NGF treatment inhibited the NGF-induced ERK phosphorylation only at two higher doses (20 and 30 μM for lanes 5 and 6 of FIG. 5, respectively).

DISCUSSION

NGF induces generation of intracellular hydrogen peroxide in PC12 cells, but the time for NGF-induced H_2O_2 production in this study differs from that reported in the earlier study.[3] Recent evidence shows that application of NGF stimulates a brief increase of intracellular ROS in PC12 cells 10 min after treatment, and this ROS is suggested to be hydrogen peroxide because NGF fails to increase the ROS in PC12 cells transfected with PS3CAT carrying human catalase.[3] In this study, our results showed that application of NGF caused a threefold increase of intracellular hydrogen peroxide in PC12 cells 1 min after treatment (FIG. 1A). This NGF-induced elevation of intracellular hydrogen peroxide decreased gradually and returned to the control level 10 min after NGF application (FIG. 1B–D). The difference in the time for NGF-induced H_2O_2 production between our study and the earlier study may result from the 10-min difference in the DCFH-DA incubation time prior to NGF application between these two studies (20 min for this study and 10 min for the earlier study). Our findings confirm that NGF stimulates the production of hydrogen peroxide in PC12 cells and support the notion that ROS in nontoxic amounts act as second messengers in a variety of signaling pathways.[20]

NGF has no effect on the intracellular level of superoxide in PC12 cells. Accumulating evidence indicates that various cytokines (e.g., interleukin 1 [IL-1] and tumor necrosis factor α [TNF-α]) and peptide growth factors (e.g., epidermal growth factor [EGF]) lead to generation of superoxide in different types of cells.[21,22] IL-1 or TNF-α stimulates release of ROS in human fibroblasts, and the released ROS has

(A)



(B)

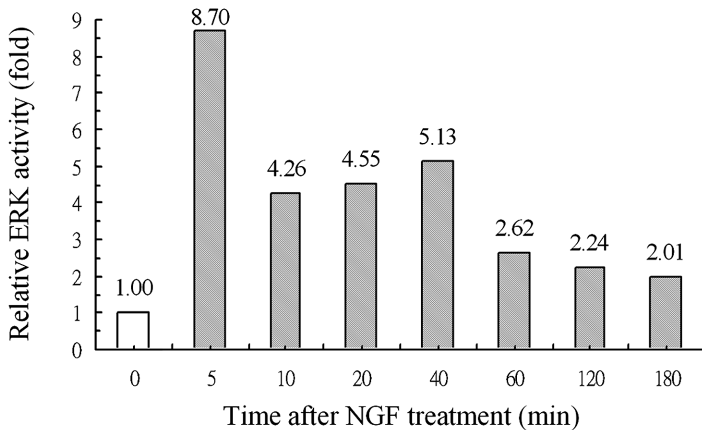


FIGURE 4. NGF induces phosphorylation of ERK in PC12 cells. (**A**) NGF (100 ng/mL) was added to PC12 cells, which were collected 5, 10, 20, 40, 60, 120, and 180 min after NGF application along with control PC12 cells. PC12 cells were then processed for detection of phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 (t-ERK1/2) by Western blotting. NGF treatment significantly increased the amount of p-ERK1/2 with a peak at 5 min after application, and the increased p-ERK1/2 lasted for at least 3 h, while keeping the t-ERK1/2 unchanged. (**B**) Amounts of p-ERK1/2 and t-ERK1/2 were quantified by a densitometer, and relative ERK activity of all treatments was calculated as described in MATERIALS AND METHODS. NGF induced a 2- to 8.7-fold increase of ERK activity in PC12 cells within 3 h after NGF application. The NGF-induced ERK activity peaked with an 8.7-fold increase at 5 min after NGF treatment, decreased to ~ 4–5 times the control value between 10 and 40 min after application, and remained at about 2 times the control value even 3 h after NGF treatment.

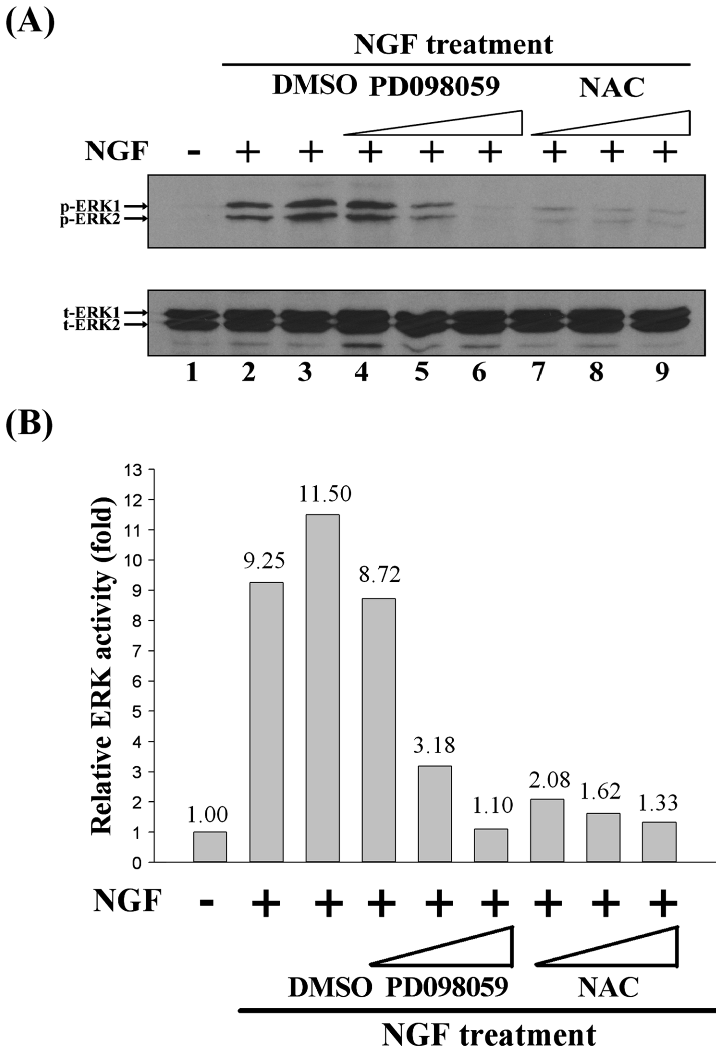


FIGURE 5. Both antioxidant *N*-acetylcysteine (NAC) and a selective MAPK inhibitor, PD098059, prevent the NGF-increased ERK phosphorylation in PC12 cells. PC12 cells were pretreated with NAC (5, 10, and 20 mM for lanes 7, 8, and 9, respectively), PD098059 (10, 20, and 30 µM for lanes 4, 5, and 6, respectively), DMSO (lane 3), or vehicle (lanes 1 and 2) for 4, 1, 1, and 1 h, respectively. Afterward, NGF (100 ng/mL) or vehicle was applied to PC12 cells, which were collected 5 min after NGF or vehicle application and processed for detection of phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 (t-ERK1/2) by Western blotting. (**A**) NGF treatment significantly increased the amount of p-ERK1/2. All three doses of NAC (lanes 7, 8, and 9) and two higher doses of PD098059 (lanes 5 and 6) suppressed the NGF-induced activation of ERK1/2. (**B**) The amounts of p-ERK1/2 and t-ERK1/2 were quantified by densitometer, and the relative ERK activity for all groups was calculated as described in MATERIALS AND METHODS. All three doses of NAC and two higher doses of PD098059 dramatically inhibited the NGF-induced phosphorylation of ERK.

been proven to be the superoxide.[21] Application of EGF to PC12 cells induces increased production of ROS, and pretreatment of PC12 cells with lipoxygenase inhibitor NDGA (inhibitor of superoxide-producing enzyme) abolishes the EGF effect on ROS production, suggesting that the ROS induced by EGF in PC12 cells is likely to be the superoxide.[22] NGF induces ROS in PC12 cells,[3] and NGF increases the generation of superoxide in neutrophils stimulated by phorbol 12-myristate 13-acetate (PMA).[23] In this study, therefore, we also examined whether NGF resulted in production of superoxide in PC12 cells. Our results indicated that NGF treatment was unable to affect the level of intracellular superoxide in PC12 cells at all time points examined (FIG. 3). Our findings fail to support the idea that the superoxide participates in NGF signaling in PC12 cells.

Application of NGF leads to a significant elevation of ERK activity in PC12 cells, and NGF-increased ERK activation is abolished by pretreatment of PC12 cells with a selective MAPK inhibitor PD098059. NGF signaling pathways include at least the Ras–Raf–MEK–MAPK pathway, PI-3 kinase pathway, and phospholipase C pathway,[13–16,24–26] and the Ras–Raf–MEK–MAPK pathway is the most well studied. Nonetheless, the detailed mechanisms of this pathway are still poorly understood. In this study, we found that NGF treatment increased the relative ERK activity in PC12 cells, which is consistent with earlier findings in sympathetic neurons and in PC12 cells.[26,27] Pretreatment with a specific MAPK inhibitor PD098059 1 h prior to NGF treatment prevented the NGF-induced ERK activation in PC12 cells, which replicates the earlier findings reported in sympathetic neurons.[26] Our findings demonstrate that ERK activation is the downstream cascade of NGF-induced MAPK signaling in PC12 cells.

Antioxidant and glutathione precursor NAC pretreatment counteracts the effects of NGF on the increase of intracellular hydrogen peroxide and the activation of ERK in PC12 cells. NAC can protect cells from oxidative damage by its direct scavenging ROS ability and by its indirect ability to increase synthesis of antioxidant glutathione.[28–30] Evidence shows that NAC inhibits the *c-fos* expression and AP-1 activation induced by NGF in PC12 cells.[31] NAC also blocks the NGF-induced MAPK activation and reduces the level of phosphorylated MAPKs in PC12 cells, which is independent of glutathione production.[31] Moreover, NAC prevents the elevated intracellular ROS, neurite extension, activation of AP-1, and phosphorylation of tyrosine in PC12 cells induced by NGF.[3] In this study, H_2O_2 application caused an increase of intracellular hydrogen peroxide, and NAC pretreatment counteracted the H_2O_2-induced production of hydrogen peroxide in PC12 cells. Most importantly, pretreatment of PC12 cells with antioxidant NAC blocked the NGF-increased generation of hydrogen peroxide and activation of ERK in PC12 cells. NAC inhibition of NGF-induced H_2O_2 production and ERK activation and suppression of NGF-induced ERK activity by a specific MAPK inhibitor PD098059 together strongly suggest that NAC inhibits the NGF-induced MAPK/ERK signaling cascades by depleting NGF-induced intracellular hydrogen peroxide.

ACKNOWLEDGMENTS

This research was supported by National Science Council grants NSC 90-2320-B-038-052 (L.Y.Y.), NSC 91-2320-B-038-010 (L.Y.Y.), and NSC 93-3112-B-038-005 (L.Y.Y.), and NSC 92-2320-B-038-055 (C.M.S.).

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