Nerve Growth Factor Treatment Prevents the Increase in Superoxide Produced by Epidermal Growth Factor in PC12 Cells*

(Received for publication, May 26, 1998, and in revised form, June 30, 1998)

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Stimulation of pheochromocytoma (PC12) cells with the mitogen epidermal growth factor (EGF) produced a rapid and robust accumulation of intracellular reactive oxygen species (ROS), an accumulation which, in other systems, has been shown to be essential for mitogenesis. Brief pretreatment of the cells with nerve growth factor (NGF) suppressed the EGF-mediated ROS increase. EGF failed to produce elevations in ROS in a PC12 variant stably expressing a dominant-negative p21ras construct (PC12-N17) or in cells pretreated with the MEK inhibitor PD98059. NGF failed to suppress the increase in ROS in the PC12 variant nnr5, which lacks p140trk receptors. The suppression of the increase in ROS by NGF was restored in nnr5 cells stably expressing p140trk (nnr5-trk), but NGF failed to prevent the increase in ROS in nrr cells expressing mutant p140trk receptors that lack binding sites for Shc and phospholipase Cγ. Among several inhibitors of superoxide-generating enzymes, only the lipoxygenase inhibitor, nordihydroguaiaretic acid reduced EGF-mediated ROS accumulation. The inhibitory action of NGF on ROS production was mimicked by the nitric oxide donor, sodium nitroprusside, and was blocked by an inhibitor of nitric-oxide synthetase, l-nitroarginine methyl ester. These results suggest a novel mechanism for the rapid interruption of mitogenic signaling by the neurotrophin NGF.

The neurotrophins are a family of polypeptides that are involved in the survival and differentiation of several different classes of neurons in both the central and peripheral nervous systems. Although some aspects of the intracellular mechanisms by which they act are now clear (1, 2), the way in which they produce global changes in phenotype, such as differentia-

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PC12 cells. Wild-type PC12 cells were loaded with the oxidant-sensitive fluorophore DCHF-DA as described under "Experimental Procedures." Images of cells were collected by laser confocal microscopy before and after treatment with EGF (5 min, 100 ng/ml), NGF (5 min, 100 ng/ml), or a combination of NGF (5 or 10 min, 100 ng/ml) and then EGF (5 min, 100 ng/ml). Images are representative of 3–5 experiments. Quantitation of ROS levels was by relative dichlorofluorescein fluorescence (scale: 0 to 255 units) and they are expressed as mean fluorescence intensity ± S.E. for 60 cells per treatment. Values in relative fluorescence units are as follows: control, 6.1 ± 1.0; EGF (5 min), 39.2 ± 1.8; PD098059 + EGF, 12.8 ± 2.5. PC12-N17: control, 3.4 ± 0.5; EGF, 8.2 ± 1.5. b, Ras dependence of NGF-induced suppression of EGF-stimulated ROS accumulation. nmr5, nmr5-Trk, and nnr-Trkdm cells were treated as described. Images are representative of duplicate experiments. Quantitation of ROS levels as described in Fig. 1. Values in relative fluorescence units are as follows: nmr5: control, 6.1 ± 0.6; EGF, 41.5 ± 2.4; NGF (10 min) + EGF, 37.7 ± 3.1. nmr5-Trk: control, 7.3 ± 0.8; EGF, 39.8 ± 3.0; NGF + EGF, 8.3 ± 1.7. nmr5-Trkdm: control, 7.7 ± 0.9; EGF, 35.4 ± 2.2; NGF + EGF, 28.5 ± 2.4.

RESULTS AND DISCUSSION

Treatment of PC12 cells with EGF produced a rapid and robust increase in ROS in the cells (Fig. 1, A and B). Treatment with NGF had no comparable effect (Fig. 1C), however pretreatment of cells for 10 min with NGF completely abolished the increase in ROS seen upon subsequent treatment with EGF (Fig. 1E).

The EGF-induced increase in ROS appears to require signaling through the Ras pathway. PD098059, a MEK inhibitor, blocked the EGF-induced increase in ROS (Fig. 2a, A–C) Ras dominant-negative cells (PC12-N17) treated with EGF did not show any increase in ROS (Fig. 2a, D and E), although, clearly, these cells have EGF receptors (11). The NGF-induced decrease in mitogen-generated ROS also appears to depend on the Ras pathway. PC12 nmr5 cells (12), which lack p140 Trk receptors for NGF, respond normally to EFG (Fig. 2b, A and B) but are not affected by pretreatment with NGF (Fig. 2b, C). When p140 Trk receptors are heterologously expressed in nmr5 cells, the ability of NGF to prevent the EGF-induced rise in ROS is restored (Fig. 2b, D and E). However PC12 nmr5 cells transfected with a mutant p140Trk, which lacks binding sites for PLCγ and for Shc (13), and thus do not show NGF-induced activation of the Ras pathway, did not show a suppressive effect of NGF on EGF-induced ROS formation (Fig. 2b, F and G). The dual reliance of EGF-induced ROS generation and NGF-induced ROS suppression in the Ras pathway is probably predictable; mitogen-induced ROS generation in fibroblasts is suppressed by the expression of dominant-negative isoforms of Ras (14), and the suppression of endogenous ROS by NGF in QT1-1 trk cells is dependent on the Ras pathway (15).

It is likely that the species of ROS generated by EGF stimulation is superoxide (∙O2−). Among a number of drugs that are known to inhibit superoxide-producing enzymes, including NADPH oxidase, cyclooxygenase, and lipooxygenase, only pretreatment of cells with the lipooxygenase inhibitor NDGA sup-
pressed EGF-induced ROS production (Fig. 3a, A–D). In addition, nitric oxide donor sodium nitroprusside (SNP) mimicked the suppressive action of NGF on ROS generation by EGF (Fig. 3b, G and H). It has been shown that nitric oxide will react with superoxide in a highly favorable reaction to produce peroxynitrite anion (OONO⁻) (16), which appears to react readily with reduced glutathione (17). It is also known that nitric oxide donor compounds inhibit lipoxigenase activity (18).

The data show that EGF treatment of PC12 cells produces a marked increase in ROS, that is likely superoxide, and that NGF produces little or none, despite the data indicating that NGF is initially mitogenic for PC12 cells (19). Pretreatment with NGF abolishes that increase in a manner dependent upon p140/trk. Further, both these actions rely on the integrity of the Ras signal transduction pathway. Finally, the inhibitory action of NGF treatment on ROS accumulation appears to involve the generation of nitric oxide and either the reaction of that nitric oxide with superoxide or the inhibition, by nitric oxide, of lipoxigenase. The acute nature of the response to NGF predicts either a very quick increase in inducible nitric-oxide synthetase or a rapid NGF-induced activation of the constitutive nitric-oxide synthetase, an effect not hitherto reported.

It is known that treatment of PC12 cells with NGF leads to a rapid down-regulation of the EGF receptor (20, 21). However, it is unlikely that this is the reason for the prevention of the ROS increase by NGF because the down-regulation is only partial and a comparable action of NGF is seen when 3T3 cells transfected with p140/trk are treated with PDGF (data not shown), and there is no reason to believe that NGF has any effect on the levels of PDGF receptors in these cells.

There is some evidence for the involvement of Ras in ROS generation. In A431 cells, overexpression of Ras was associated with a 40-fold induction in the promoter activity of human 12-lipoxygenase (22). Arachidonic acid metabolites produced by lipoxigenase have been reported to be Ras-dependent mitogens for certain cells (23) and may also influence Ras-dependent proliferation by modulating its associations with GTPase-activating proteins (24, 25). There is only a limited amount of information regarding the involvement of Ras in the regulation of nitric oxide production; lipopolysaccharide induction of nitric-oxide synthetase in astrocytes can be prevented with specific Ras inhibitors (26).

The generation of ROS is an essential element in signaling from mitogen receptors (3, 4). If increases in ROS are prevented the cells neither proliferate nor express normal markers of PDGF-induced mitogenic signaling. Treatment of appropriate cells with neurotrophins inhibits their cell division. The present data indicate that this effect of the neurotrophins is, at least in part, due to their ability to generate nitric oxide which, in turn, quenches the ROS generated by mitogen treatment and thus interrupts the signal transduction pathway that leads to cell division.

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