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Enhancement or Induction of Neurite Formation by a Protein Tyrosine Phosphatase Inhibitor, 3,4-Dephostatin, in Growth Factor-Treated PC12h Cells

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We studied the effect of the 3,4-dihydroxy analogue of dephostatin (3,4-dephostatin), an inhibitor of protein-tyrosine phosphatase (PTPase), on the differentiation of rat pheochromocytoma PC12 cells. 3,4-Dephostatin accelerated NGF-induced neurite formation in PC12h cells, a subline of PC12 cells, whereas the inhibitor alone did not induce neurite formation. It sustained the NGF-induced tyrosine phosphorylation of several proteins, most prominently that of mitogen-activated protein (MAP) kinase. EGF alone did not induce differentiation in PC12h cells, but it induced neurite formation in the presence of 3,4-dephostatin. The inhibitor also prolonged EGF-induced tyrosine phosphorylation and activation of MAP kinase. An inactive analogue of dephostatin, 2′-*O*-methyl-dephostatin showed no effect on either neurite formation or MAP kinase tyrosine phosphorylation in NGF- or EGF-treated PC12h cells. Thus, we demonstrated that the PTPase inhibitor could enhance growth factor-induced differentiation in PC12 cells possibly by sustaining the MAP kinase activity.

The rat pheochromocytoma PC12 cell line was originally isolated as a nerve growth factor (NGF)-responsive one and has been much used as a model of NGF-induced differentiation (1). There are two types of receptors for NGF: one is a high-affinity receptor; and the other, a low-affinity one. The high-affinity receptor consists of a 75K protein and 140K c-Trk (2). NGF stimulates the intrinsic protein-tyrosine kinase of c-Trk to undergo tyrosine autophosphorylation. This c-Trk and other protein kinases in the down-stream are considered to regulate mitogen-activated protein (MAP) kinase (3) and other enzymes such as phospholipase Cγ (4) in PC12 cells.

PC12 cells also possess the epidermal growth factor (EGF) receptor. EGF has a rather proliferative effect on PC12 cells, and does not induce differentiation (5). However, many of the initial responses are commonly induced by both NGF and EGF, such as activation of the intrinsic protein-tyrosine kinase activities of their receptors and increased activation of the MAP kinase cascade (6). To date, although the exact biochemical differences that account for the final biological effects of NGF and EGF have not been defined, an important difference between these two growth factors has been implied from the time course of the tyrosine phosphorylation cascade including MAP kinase. Activation of MAP kinase by NGF is sustained at a high level for several hours, whereas the activation by EGF is transient (7).

Cellular tyrosine phosphorylation should be regulated by both protein-tyrosine kinases and protein-tyrosine phosphatases (PTPases). If prolonged tyrosine phosphorylation is essential for differentiation of PC12 cells, inhibition of PTPases may stimulate or induce differentiation in NGF- or EGF-treated PC12 cells.

Previously we isolated dephostatin from *Streptomyces* as an inhibitor of CD45 PTPase of human T cell leukaemia cells (8,9). Total synthesis of dephostatin was also achieved (10). Dephostatin is a competitive inhibitor in the PTPase reaction but does not inhibit serine/threonine phosphatases 2A and 2B (11). Since dephostatin is unstable and rapidly degraded, we also synthesized its stable analogue, the 3,4-dihydroxy form of dephostatin (3,4-dephostatin) (12). It also inhibits CD45 PTPase competitively with the substrate, as does dephostatin, with an IC_{50} value of about 3 μg/ml. We also prepared an inactive analogue of dephostatin, 2′-*O*-methyl-dephostatin (12), as shown in Fig. 1. In this report we studied the effect of 3,4-dephostatin on neurite formation and MAP kinase activation in both NGF-treated PC12h cells and EGF-treated ones.

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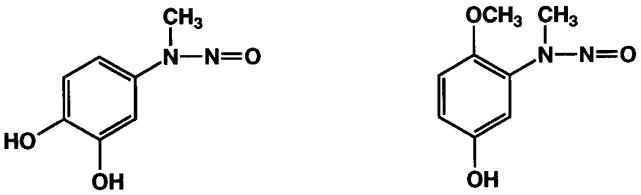

FIG. 1. Structures of dephostatin analogues.

MATERIALS AND METHODS

Materials. The analogues of dephostatin (Fig. 1) were synthesized as described previously (12). NGF (2.5S) and EGF were obtained from Sigma and Biomedical Technologies Inc., respectively. Anti-phosphotyrosine antibody (4G10) and anti-ERK2 antibody (B9) were purchased from Upstate Biochemicals, Inc.; and biotinylated anti-mouse secondary antibody and streptoavidin-alkaline phosphatase conjugate (SAAP), from Amersham.

Cell culture. PC12h cells were kindly supplied by Prof. H. Hatanaka, Osaka University. We employed this cell line because of less spontaneous differentiation in it. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% semi-fetal calf serum (SFCS) (Mitsubishi-Kagaku).

Neurite outgrowth assay. PC12h cells were seeded into collagen-coated 24-well culture dishes at a density of $1.5\text{-}2\times10^4$ cells/well. The cells were first grown in complete medium for 24 h, rinsed once with serum-free DMEM, and then cultured in DMEM containing 1% SFCS for an additional 24 h. NGF (50 ng/ml) or EGF (10 ng/ml) was added to the medium in the presence or absence of the desired concentrations of the dephostatin analogues. After selected incubation periods, cells possessing one or more neurites of a length more than 2 times the diameter of the cell body were scored as positive. About 600 cells were scored in each well (200 cells in each of 3 random microscopic fields), and the percentage of cells with neurites was calculated as the differentiation ratio.

Western blotting. PC12 cells were grown to 70-80% confluence in 60-mm tissue culture dishes. After incubation for 24 h in 1% SFCS DMEM, the cells were treated with the growth factors and/or chemicals for various times. Thereafter they were washed with ice-cold Dulbecco's phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium vanadate and dissolved in RIPA buffer (25 mM HEPES, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5 M sodium chloride, 5 mM EDTA, 50 mM sodium fluoride, 100 mM sodium vanadate, 1 mM PMSF, 0.1 mg/ml leupeptin [pH 7.8]. After solubilization, the protein content was assayed with a Bio-Rad protein assay kit, and 15 μg of proteins was subjected to SDS-PAGE using 10% gels. The proteins were transferred electrophoretically to a nitrocellulose membrane, which was then soaked in TBS-Tween buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 0.1% Tween 20) with 6% calf serum. After incubation with anti-phosphotyrosine or anti-ErK2 antibody, the membrane was incubated with biotinylated anti-mouse secondary antibody. Proteins were visualized by use of the SAAP staining system.

RESULTS

3,4-Dephostatin accelerates NGF-induced neurite formation. NGF induced morphological differentiation in PC12h cells time-dependently in 1-3 days. As shown in Fig. 2, 3,4-dephostatin enhanced NGF-induced neurite formation at 0.1-1 μg/ml in a dose-dependent manner. This enhancing effect of 3,4-dephostatin was observed during the first 2 days. However, after 3 days, the neurite formative effect of NGF alone reached a plateau, and the effect of 3,4-dephostatin was no longer seen, suggesting that 3,4-dephostatin accelerated NGF-induced neurite formation. 3,4-Dephostatin alone did not induce neurite formation, nor did 2′-*O*-methyl-dephostatin accelerate NGF-induced neurite formation in PC12h cells.

3,4-Dephostatin sustains the tyrosine phosphorylation of MAP kinase in NGF-treated PC12h cells. As shown in Fig. 3, exposure of PC12h cells to NGF (50 ng/ml) for 5 min resulted in tyrosine phosphorylation of several proteins including the two predominant proteins at 42 and 44 kDa, as detected by Western blotting analysis; and the levels of these two proteins decreased thereafter to almost that of the untreated cells within 3 hours. At 0.3-3 μg/ml, 3,4-dephostatin dose-dependently sustained the NGF-induced tyrosine phosphorylation of these two proteins, as shown in Fig. 3. In the presence of 3 μg/ml 3,4-dephostatin, the phosphorylated 42- and 44-kDa proteins were detectable even after 3 hours. Since these two proteins are likely to be isoforms of MAP kinase (Erk1 and 2), activation of MAP kinase was assessed by anti-Erk2 antibody. As shown in the lower panel of Fig. 3, the NGF-induced activation of MAP kinase, which appeared as an up-

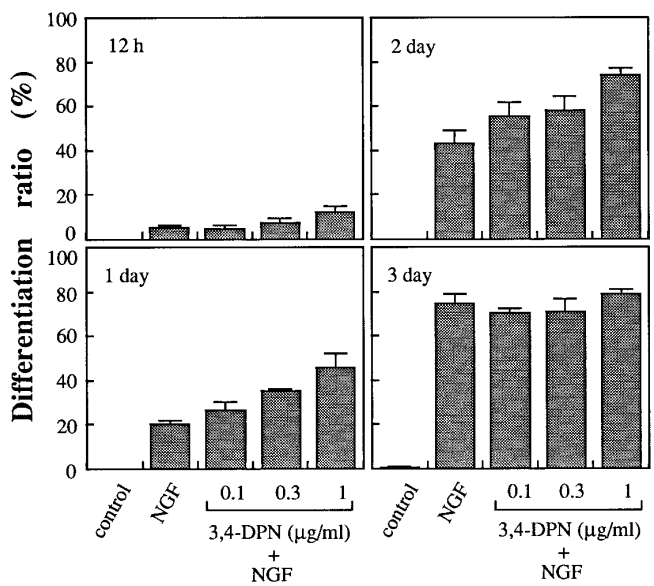

FIG. 2. Time course and dose-dependent effect of 3,4-dephostatin on NGF-induced neurite formation in PC12h cells. PC12h cells were treated with NGF (50 ng/ml) either alone or in combination with 3,4-dephostatin as indicated. At the indicated times, about 600 cells were scored, and the differentiation ratio was calculated as described under "Materials and Methods." Values are means ± SD of triplicate determinations.

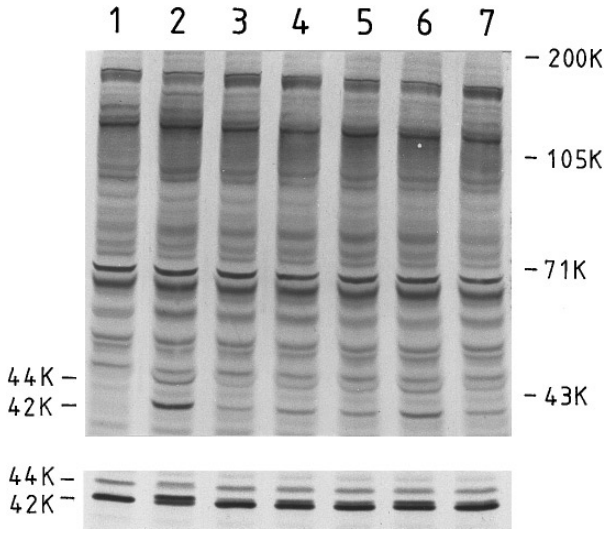

FIG. 3. Tyrosine phosphorylation and activation of MAP kinase induced by NGF in the presence of 3,4-dephostatin. PC12h cells were left untreated (lane 1) or were treated with 50 ng/ml of NGF (lanes 2-7) for 5 min (lane 2) or 3 h (lane 3-7). 3,4-Dephostatin was added with NGF at 0 (lane 3), 0.3 (lane 4), 1.0 (lane 5), or 3.0 (lane 6) μg/ml. The cells were incubated with NGF and 3 μg/ml of 2′-*O*-methyl-dephostatin (lane 7). The lysates were analyzed by Western blotting using anti-phosphotyrosine antibody (*upper panel*) and anti-Erk2 antibody (*lower panel*). Activation of the 42K MAP kinase is indicated by the upward band shift in the *lower panel.*

shifted band of 42K, was enhanced in the cells treated with both NGF and 3,4-dephostatin. On the other hand, 2′-*O*-methyl-dephostatin, an inactive analogue, did not alter NGF-induced tyrosine phosphorylation or activation of MAP kinase. A slightly higher concentration (3 μg/ml) of 3,4-dephostatin was necessary to sustain the phosphorylation, presumably because a larger population of the cells was used for the Western blotting analysis than for the morphological assay. We confirmed that 3,4-dephostatin alone did not induce tyrosine phosphorylation of MAP kinase up to 3 μg/ml (data not shown).

EGF induces neurite formation in the presence of 3,4-dephostatin. Unlike NGF, EGF at 10 ng/ml did not induce prominent neurite formation in PC12h cells. However, as shown in Fig. 4, EGF induced marked neurite formation in the presence of 1 μg/ml 3,4-dephostatin. The time course of the differentiation ratio is shown in Fig. 5. The stimulating effect of 3,4-dephostatin was marked during the first 2 days and seemed to saturate thereafter. Simultaneous treatment with EGF and 2′-*O*-methyl-dephostatin did not have any effect on PC12h cells.

3,4-Dephostatin sustains the tyrosine phosphorylation of MAP kinase in EGF-treated PC12h cells. When PC12h cells were treated with 10 ng/ml of EGF, several proteins, most prominently the two isoforms of MAP kinase, underwent tyrosine phosphorylation within 5 minutes, as shown in Fig. 6. EGF-induced tyrosine phosphorylation of MAP kinase decreased more rapidly after 5 min than that induced by NGF, and within 1 h the level of phosphorylation decreased to almost that of the untreated cells. As shown in Fig. 6, simultaneous treatment with EGF and 3,4-dephostatin at 0.3-3 μg/ml resulted in longer duration of MAP kinase tyrosine phosphorylation and activation, which remained detectable after 1 h and was dose dependent. On the other hand, 2′-*O*-methyl-dephostatin, at the highest concentration used for 3,4-dephostatin, did not significantly sustain EGF-induced tyrosine phosphorylation of MAP kinase (data not shown).

DISCUSSION

3,4-Dephostatin was found to be stable for 12 hours in DMEM, when extracted and assayed by high-pressure liquid chromatography. It inhibited the growth of PC12h cells in DMEM containing 10% SFCS with the IC_{50} value of 2.5 μg/ml. In the present study, 3,4-dephostatin, which inhibits PTPase, accelerated neurite formation in NGF-treated PC12h cells, and also sustained NGF-induced tyrosine phosphorylation of MAP kinase in the cells. In our assay system, MAP kinases were strongly tyrosine-phosphorylated by NGF compared with other proteins. These results suggest that PTPases are involved in controlling the tyrosine phosphorylation and activation of MAP kinase to regulate the rate of neurite formation. The relation between sustained MAP kinase activation and rapid neurite induction in NGF-treated PC12 cells was earlier demonstrated by several groups: Hempstead *et al.* observed the rapid neurite induction and the sustained MAP kinase activation in PC12 cells overexpressing Trk (13), and Yamada *et al.* reported the prolonged tyrosine phosphorylation of MAP kinase in a subline of PC12 cells responding rapidly to NGF (14). In addition, PC12 cells exhibited neuronal differentiation when the MAP kinase cascade was constitutively activated (15). Therefore, our results are consistent with these observations on the role of MAP kinase in differentiation.

Dibutyryl cAMP also potentiated neurite formation in PC12h cells, but neither tyrosine phosphorylation nor activation of MAP kinase was observed during this differentiation (16). As expected, 3,4-dephostatin when added with dibutyryl cAMP did not alter the MAP kinase tyrosine phosphorylation and activation, nor did it affect neurite formation (data not shown). Thus it is unlikely that 3,4-dephostatin affected MAP kinase-independent pathway in NGF- or EGF-treated PC12h cells.

Another known PTPase inhibitor, vanadate was reported to rather inhibit NGF-induced differentiation (17). In our assay system, vanadate also inhibited the NGF-induced neurite formation. It may be possible

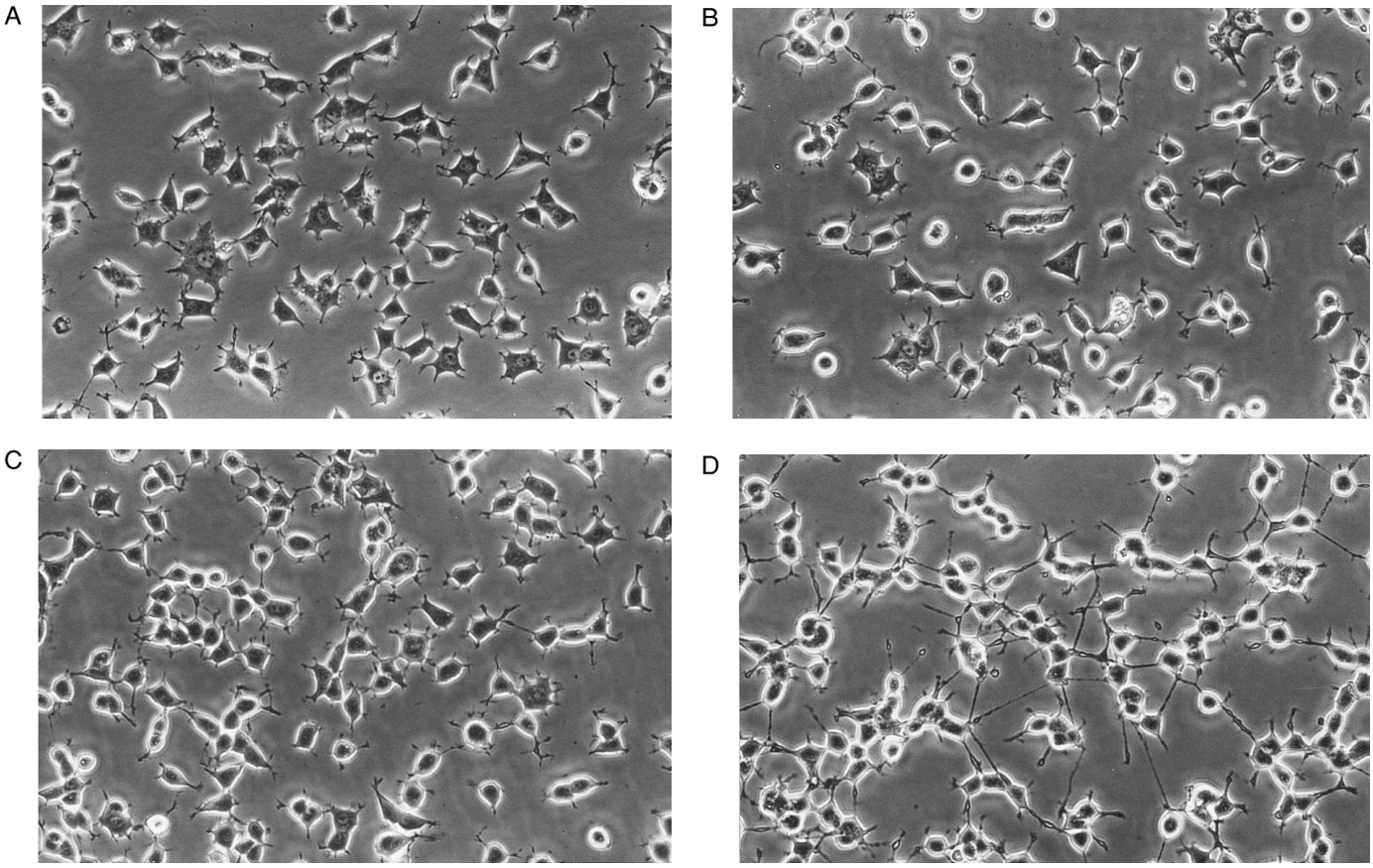

FIG. 4. Induction of neurite formation by EGF in PC12h cells in the presence of 3,4-dephostatin. PC12h cells were left untreated (A) or treated with EGF (10 ng/ml) (B), 3,4-dephostatin (1 μg/ml) (C), or EGF and 3,4-dephostatin (D) for 2 days.

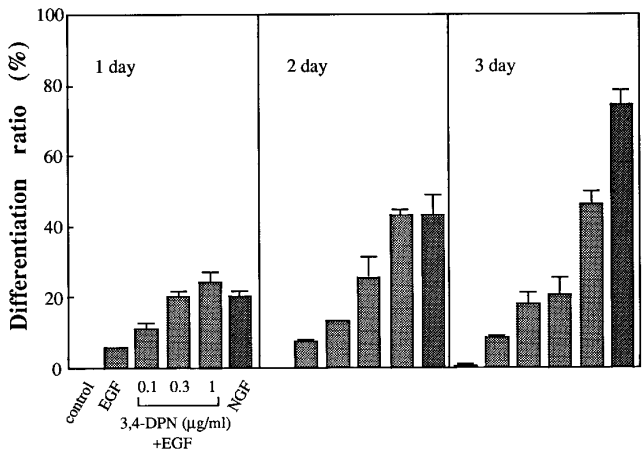

FIG. 5. Time course and dose-dependent effect of 3,4-dephostatin on neurite formation in EGF-treated PC12h cells. PC12h cells were untreated (control) or treated with EGF (10 ng/ml) in the absence or presence of 3,4-dephostatin as indicated. At the indicated days, about 600 cells were scored, and the differentiation ratio was calculated. NGF was added at 50 ng/ml for comparison. Values are means ± SD of triplicate determinations.

that 3,4-dephostatin and vanadate have a different inhibitory spectra.

PC12 cells possess several growth factor receptors, such as EGF and insulin receptors, that do not potentiate the differentiative signal. However, overexpression of these receptors leads to EGF- and insulin-dependent differentiation accompanied by sustained activation of MAP kinase (18,19). These observations are consistent with our results that 3,4-dephostatin sustained EGF-induced tyrosine phosphorylation of MAP kinase and converted the response to EGF to a differentiative one in PC12h cells. Moreover, the magnitude of the neurite proliferative effect produced by both EGF and 3,4-dephostatin was comparable to that induced by NGF alone during the first 2 days. We thus demonstrated that a PTPase inhibitor of low molecular weight could potentiate EGF to induce differentiation in intact PC12 cells.

One possible candidate target of 3,4-dephostatin may be MAP kinase phosphatase, although activation of c-Trk or EGF receptor can not be ruled out. MAP kinase phosphatase is a subfamily of dual specificity protein tyrosine phosphatases (dsPTPs) that specifically inactivate MAP kinase by dephosphorylating it on both tyro-

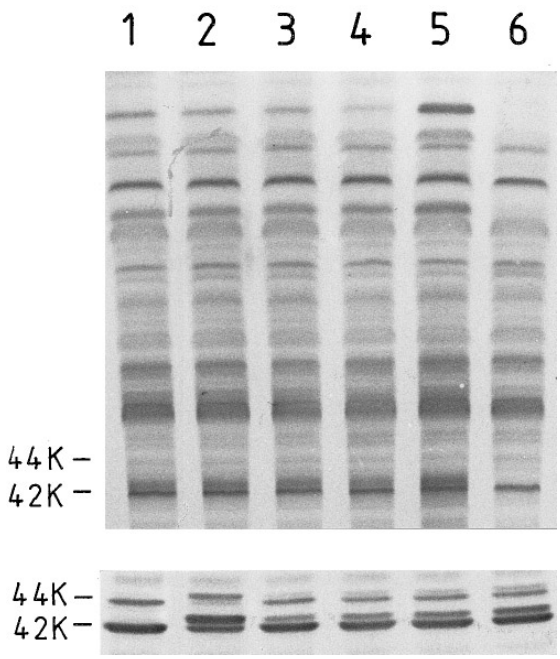

FIG. 6. Tyrosine phosphorylation and activation of MAP kinase induced by EGF in the presence of 3,4-dephostatin. PC12h cells were left untreated (lane 1) or were treated with 10 ng/ml of EGF (lanes 2-6) for 5 min (lane 2) or 1 h (lanes 3-6). 3,4-Dephostatin was added with NGF at 0 (lane 3), 0.3 (lane 4), 1.0 (lane 5), or 3.0 (lane 6) μg/ml.

sine and threonine residues (20-23). Further investigation will be required to address the critical target of 3,4-dephostatin.

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