신경성장기전 및 치료제개발

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ABSTRACT

Regulation of nerve growth factor (NGF)-induced neuronal differentiation by GTPase activating protein(GAP) and its mechanism were investigated in rat pheochromocytoma cell line, PC12. Overexpression of GAP caused the delay in the onset of neurite outgrowth of PC12 cells in response to NGF. GAP has been known to inhibit $p21^{ras}$, the activated form of which induces neuronal differentiation. Therefore, the activity of $p21^{ras}$ was compared in control cells and cells overexpressing GAP indirectly by measuring the activities of B-Raf and MAP kinase that are known to be positively regulated by $p21^{ras}$. Surprisingly, NGF-induced activities of these two proteins were the same in control cells and GAP-overexpressing cells.

Activities of Trk, PLC-r and SHC that act at a site upstream to $p21^{ras}$ in NGF signal transduction pathway were not also affected by GAP overexpression.

Interestingly, however, the extent of tyrosine phosphorylation of SNT was found to be remarkably low in cells overexpressing GAP. It has been shown previously that neurotrophins and not mitogens induce SNT tyrosine phosphorylation in PC12 cells. Thus it is possible that the timing of NGF-induced neuronal differntiation may be in part regulated by SNT and the slower onset of neurite outgrowth in cells overexpressing GAP may be through the inhibition of SNT by GAP

In addition to NGF, there are also several other factors that are members of a neurotrophin family. They include BDNF, NT3, and NT4/5. These factors show some preferences for their receptor types; for instance, BDNF and NT3 bind to TrkB and TrkC, respectively. The diversity of factors and their receptors led us to study the mechanisms of action of two agents(K252b and retinoic acid) that have

neurotrophic activities toward some neuronal cells. These two agents differed in their mechanisms of action. K252b potentiated NT3-induced activation of TrkC, while retinoic acid seemed to induce the expression of TrkB.

INTRODUCTION

Modern society faces an increase in the incidence of neurological disorders and accidents. A key to properly treat these problems would be to first understand the pathology of these disorders. Biochemical knowledge on the normal cell physiology of the nerve cells is fundamental in understanding the pathology of the disorders. Furthermore, development of therapeutic agents that possess neurotrophic activity requires a full characterization of the mechanisms of action of these agents and thus is aided by basic reserches on nerve cells.

Nerve cells require neurotrophins for their survival and maintenance. NGF, a member of the neurotrophin family, is necessary for the survival and maintenance of subpopulations of neurons. Most of the studies elucidating the mechanism of action of NGF have been obtained using the rat pheochromocytoma cell line, PC12. NGF induces PC12 cells to differentiate into a neuronal phenotype characterized by the extension of neurites, generation of a sodium-based action potential, and the cessation of cell division. NGF binds to two cellular receptors, the Trk tyrosine kinase and $p76^{NGFR}$. While the role of p75 in transmitting NGF signals is unclear, Trk has been shown to regulate the timing and the extent of NGF-induced neurite outgrowth. Like many other receptor tyrosine kinases, the transphosphorylation activity of Trk is activated by ligand binding. The activated receptor Trk stimulates the tyrosine phosphorylation of several signal transducing proteins in PC 12 cells, including phospholipase C-gammal and SHC. Trk also mediates the activation of the serine/threonine kinases, PKC, B-Raf, and MAP kinases. These proteins mentioned here, however, are activated also by mitogenic factors. Recently, one protein named SNT was discovered that responds to the differentiation-specific factors

only. SNT undergoes tyrosine phosporylation in response to NGF or FGF and not EGF.

Another important component of the NGF signal transduction pathway is $p21^{ras}$, $p21^{ras}$ exists in GTP-bound active or GDP-bound inactive form. The activity of $p21^{ras}$ is attenuated by GAP. GAP stimulates the low intrinsic GTP ase activity of the $p21^{ras}$, converting this protein to an inactive GDP-bound state. In addition to this negative effect on $p21^{ras}$, GAP has been shown to play a role as an effector of p21rasin other systems. Thus, the role of GAP in NGF-induced neuronal differentiation of PC12 cells was examined.

Here we also present the results from the studies on the mechanisms of two agents (K252b retinonic acid) that affect the differentiation of nerve cells.

RESULTS AND DISCUSSION

1)Mechanism of NGF Action on PC 12 Cells

In order to study the function of GAP in PC12 cells, the amount of GAP in the cell was increased by transfecting the cells with the plasmid containing GAP cDNA. Through the transfection and screening precedures, two clones were selected which exhibited a 2-3 fold increase in the level of GAP expression or activity. When NGF-induced neurite outgrowth was assessed, it was observed that these two clones extended neurites in a manner different from the control cells. The onset of neurite outgrowth was displayed on GAP-overexpressing cells: they took about 5 days to show visible neurites, while control cells required 2 days. Since FGF also promotes the neuronal differentiation of PC 12 cells, we asked whether GAP-overexpressing cells exhibited slower neurite outgrowth responses to FGF.

FGF-treated GAP-overexpressing cells behaved identically to FGF-treated control cells in their neurite outgrowth responses. Thurs, overexpression of GAP in PC cells delayed the appearance of only NGF-and not FGF-induced neurites.

Next, we attempted to investigate the mechanism of this effect of GAP on

NGF-induced neuronal differentiation of PC 12 cells. Since this negative effect of GAP could be through the inhibition of $p21^{ras}$ function, activities of the proteins known to be regulated by $p21^{ras}$ were assayed. Activities were determined by assessing the level of autophosphorylation of B-Raf or tyrosine phosphorylation of MAP kinase. Either the level of autophosphorylation of B-Raf or tyrosine phosphorylation of MAP kinase was similar in both control cells and cells overexpressing GAP. These results indicate that B-Raf and MAP kinase activities were not affected by GAP overexpression, and thus suggest that the change in phenotype caused by GAP overexpression was not due to the modulation of $p21^{ras}$ by GAP.

To rule out the possibility that GAP overexpression could affect the activities of proteins that are upstream to $p21^{ras}$ in NGF signal transduction pathway, the activities of Trk, PLC-gamma1, and SHC were assayed. NGF-induced activation of these proteins was the same in control cells and cells overexpressing cells.

GAP overexpression did not alter the activity of several components of the signal transduction pathway involving $p21^{ras}$, suggesting that GAP may delay the differentiation of PC 12 cells by $p21^{ras}$-independent pathways. A candidate target of GAP activity on PC 12 cells is SNT. SNT is rapidly phosphorylated on tyrosine in response to differentiation factors but not mitogens for PC 12 cells. NGF-induced tyrosine phosphorylation of SNT is dependent upon the activation of Trk but not $p21^{ras}$. Thus SNT appears to function distally to Trk but proximally to, or in a parallel signal transduction pathway to $p21^{ras}$. SNT tyrosine phosphorylation was asayed in control cells or GAP-overexpressing cells treated with NGF. While SNT tyrosine phosphorylation was stimulated by NGF in both control cells and cells overexpressing GAP, the extent of this increase was 5 to 12 fold less in GAP-overexpressing cells. Since FGF induced the outgrowth of neurites from control cells overexpressing GAP, we examined the effect of FGF on tyrosine phosphorylation of SNT. FGF stimulated similar levels of SNT tyrosine phosphorylation in control and GAP-overexpressors. These results show a correlation between the effect of GAP overexpression on neuronal

differentiation and its effect on the extent of SNT phosphorylation.

Our results suggest that GAP might regulate the timing of NGF-induced neurite formation in PC 12 cells through the inhibition of SNT.The dependence of NGF-mediated SNT tyrosine phosphorylation on the level of GAP expression,and the apparent independence of SNT tyrosine phosphorylation from $p21^{ras}$activity suggest that GAP has distinct biochemical functions in PC 12 cells.

2)Mechanisms of Actions of Chemicals Affecting Neurotrophin Signal Transduction Pathway

The fact that as can be noticed above,a number of proteins involved in NGF action are protein kinases has led researchers to study the effects of several pretein kinase inhibitors on neurotrophin-induced neuronal differentiation. One such compound, K252b has been found to block NGF action on PC 12 cells. K252b is an alkaloid materal obtained from the culture broth of Nocardiopsis sp. Although K252b inhibits the activity of protein kinase C *in vitro*, it does not penetrate through the cell membrane. This suggests that the site of K252b action is probably on the plasma membrane. Thus, we determined whether K252b could affect the activity of TrkA which as a NGF receptor, possesses tyrosine kinase activity. K252b inhibited the tyrosine phosphorylation of TrkA. However, when we tested whether this inhibition was specifically on TrkA or on other types of Trk receptors, it was found that K252b activated NT3-induced tyrosine phosphorylation of TrkC. NT3 is also a member of neurotrophin family and binds to TrkB,a subtype of Trk, while NGF binds to TrkA. Therefore, it seems that K252b can have either negative or positive effects on the receptor tyrosine kinase activities, depending on the receptor types.

Retinoic acid(RA) also has been known to induce neurotrophin responsiveness in sympathetic neurons, suggesting that RA may stimulate the expression of neurotrophin receptors. Thus we compared the levels of expression of TrkB receptor from the SY5Y neurobalstoma cells treated with RA for different length of days. It was observed that the expression of TrkB was induced in cells treated with RA and the extent of induction was higher in cells treated for

longer periods than in cells treated for shorter periods.

The results from these experiments indicate that K252b and RA differ each other in their mechanisms of action: K252b acts on plasma membrane, whereas the site of action of RA is probably inside the cell.