

Nerve growth factor-induced expression of the GTP cyclohydrolase I gene via Ras/MEK pathway in PC12D cells

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Abstract

Neurotrophins are essential for the development and survival of the catecholaminergic neurons. GTP cyclohydrolase I (GCH) is the first and rate-limiting enzyme in the biosynthesis of 5,6,7,8-tetrahydrobiopterin (BH4), the required cofactor for tyrosine hydroxylase. Previously, we reported that TH requires the Ras/mitogen-activated protein kinase kinase (MEK) pathway for its induction by nerve growth factor (NGF). Here, we examined intracellular signals required for NGF-induced expression of the GCH gene in PC12D cells. The activity of GCH was increased up to 5-fold after the NGF treatment, and the increase was repressed by pretreatment with U0126, an MEK1/2 inhibitor, but not with protein kinase A (PKA),

phosphoinositide 3-kinase (PI3K), p38 mitogen-activated protein kinase (MAPK), and c-Jun NH₂-terminal kinase (JNK) inhibitors. Induction of GCH mRNA by NGF was also abolished by pretreatment with U0126. The human GCH promoter activity was significantly enhanced by NGF treatment. Deletion analysis showed that the 465-bp 5'-flanking region is responsible for NGF-enhanced promoter activity. These data suggest that the Ras–MEK pathway is required for coordinate expression of the GCH and TH genes induced by neurotrophins.

Keywords: GTP cyclohydrolase I, mitogen-activated protein kinase kinase, nerve growth factor, PC12D, Ras, tyrosine hydroxylase.

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GTP cyclohydrolase I (GCH; EC 3.5.4.16) is the first and rate-limiting enzyme in the biosynthesis of 5,6,7,8-tetrahydrobiopterin (BH4) (Nichol *et al.* 1985). Various extracellular stimuli induce the expression of the GCH gene in neural, lymphocytic and endothelial cells (Anastasiadis *et al.* 1996; D'Sa *et al.* 1996; Anastasiadis *et al.* 1998), and cellular BH4 content is mainly regulated by the GCH activity. BH4 is the required cofactor for three aromatic amino acid monooxygenases, i.e. tyrosine (TH; EC 1.14.16.3), tryptophan (TPH; EC 1.14.16.4), and phenylalanine hydroxylase (PAH; EC 1.14.16.1), and the family of nitric oxide synthases (NOS; EC 1.14.13.39) (Kaufman 1993). The aromatic amino acid mono-oxygenases are essential for synthesizing hormones and neurotransmitters such as dopamine, norepinephrine, epinephrine, serotonin, and melatonin. The nitric oxide synthase generates nitric oxide, which is a regulatory factor for various physiological processes in the nervous, vascular, and immune systems. Biosynthesis of catecholamines (CA) is mainly regulated by TH in catecholaminergic neurons (Nagatsu *et al.* 1964). The TH activity is tightly regulated by several factors at transcription, translation and post-translation levels. BH4 is an essential cofactor, not only for the TH activity, but also for TH protein level (Sumi-Ichinose *et al.* 2001). GCH and TH

genes are often co-induced in cell lines including PC12 cells and primary cultured sympathetic neurons (Bauer *et al.* 2002; Suzuki *et al.* 2002a). In addition, suppression of GCH expression and BH4 content was demonstrated during developmental change in neurotransmitter phenotype noradrenergic to cholinergic, suggesting that GCH expression can be controlled independently of TH expression (Habecker *et al.* 2002). In view of this, the expression control of the GCH is very important in catecholaminergic neurons.

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Abbreviations used: ATF, activating transcription factor; BH4, 5,6,7,8-tetrahydrobiopterin; CA, catecholamine; CRE, cAMP response element; Erk, extracellular signal-regulated kinase; GCH, GTP cyclohydroxylase I; JNK, c-Jun NH₂-terminal kinase; MEK, mitogen-activated protein kinase kinase; NGF, nerve growth factor; NOS, nitric oxide synthase; PAGE, polyacrylamide gel electrophoresis; PAH, phenylalanine hydroxylase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; SDS, sodium dodecyl sulfate; SPR, sepiapterin reductase; TH, tyrosine hydroxylase.

Cyclic AMP-elevating hormones and reagents, and derivatives of cAMP enhance GCH expression in PC12 cells and cultured dopaminergic neurons (Zhu *et al.* 1994; Anastasiadis *et al.* 1998) as well as TH expression. The rat GCH gene proximal promoter has a non-canonical cAMP response element (CRE) and an adjacent perfect CCAAT box, which are both required for maximal and cell type-specific cAMP-dependent transcription (Kapatos *et al.* 2000). The rat and human GCH proximal promoters exhibit almost 80% sequence homology (Kapatos *et al.* 2000) and the location of this CRE and CCAAT-box are conserved in the human gene. Like the rat and mouse genes (Shimoji *et al.* 1999; Kapatos *et al.* 2000), the 5'-flanking region of the human GCH gene also supports transcription in transient transfection assays of reporter-gene constructs (Witter *et al.* 1996; Hirayama *et al.* 2001; Suzuki *et al.* 2002a).

Nerve growth factor (NGF), which is the prototype of the neurotrophin family, is shown to be required for the development and survival of peripheral sympathetic neurons at late embryonic and post-natal stages using gene-targeted mutant mice of NGF and its receptor tyrosine kinase, TrkA (Crowley *et al.* 1994; Smeyne *et al.* 1994; Snider 1994; Fagan *et al.* 1996; Patel *et al.* 2000). NGF has been demonstrated to up-regulate the TH gene expression in cultured peripheral sympathetic and sensory neurons, and PC12 and its subclonal cell lines. The PC12 cell line is a useful model of sympathetic neuronal differentiation induced by NGF.

In PC12 cells, NGF activates multiple intracellular signals including Ras/mitogen-activated protein kinase kinase (MEK) and PI3K/Akt pathways (Friedman and Greene 1999). Both of these pathways are thought to be involved in cell survival, and neuronal gene expression in response to NGF (Friedman *et al.* 1999; Bibel and Barde 2000). We previously showed that TH gene transcription induced by NGF stimulation required the Ras/MEK pathway, in PC12D cells (Suzuki *et al.* 2004), a subclone of the PC12 cell line (Kato-Semba *et al.* 1987).

NGF has been reported to up-regulate BH4 content and GCH activity (Suzuki *et al.* 1988; Hirayama and Kapatos 1995; Anastasiadis *et al.* 1996, 1997). GCH and TH mRNAs were increased by treatment with NGF in cultured superior cervical ganglia, suggesting that the increase of BH4 by GCH expression is required for induction of TH activity by NGF (Hirayama *et al.* 1995). The increase of BH4 by NGF is also suggested to be essential for cell proliferation by an unknown mechanism (Anastasiadis *et al.* 1997). However, the critical intracellular signaling pathway to induce GCH expression by NGF is as yet unidentified.

In the present study, we demonstrated that NGF-induced GCH activity and gene expression were mediated by the Ras/MEK pathway using PC12D cells. We also demonstrated that the 5'-flanking region of the GCH gene is responsible for NGF-enhanced GCH promoter activity.

Materials and methods

Cell culture

PC12D cells (Kato-Semba *et al.* 1987) were provided from Dr Yamakuni (Tohoku University), and grown in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) containing 5% fetal bovine serum (Equitech-Bio Inc., Kerrville, TX, USA), and 10% horse serum (JRH Biosciences, Lenexa, KS, USA). Cells were incubated at 37°C in 5% CO₂ in air, and the medium was changed every 3–4 days. Cells were passaged when 80–90% confluent.

Measurement of GCH activity

Cells were washed twice and suspended with 100 mM Tris-HCl (pH 8.0) buffer. To prepare cell lysates for the measurement of enzymatic activities, the suspensions were freeze-thawed at –80°C and centrifuged at 15 000 g for 10 min at 4°C; and then the supernatants were immediately collected as the cell lysates. GCH activity was assayed as described previously (Suzuki *et al.* 2002a).

Quantitative real-time PCR analysis

Total RNA was isolated from each clone by using the TRIZOL reagent (Gibco, Rockville, MD, USA). The total RNA was subjected to reverse transcription by using Superscript II (Gibco). Analysis of GCH transcripts by quantitative real-time PCR was performed on a LightCycler using a LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). MgCl₂ was added to a final concentration of 4 mM, and two oligonucleotide primers, 5'-AGCATCACCTGGTCCCATTTG-3' (forward) and 5'-TTCCACAATCTGGCAAGTT TG-3' (reverse), were added to a final concentration of 500 nM each. In parallel, we analyzed the 18S rRNA as an internal control for normalization. Real-time PCR of 18S rRNA was performed on an ABI PRISM 7700 using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA).

DNA transfection and luciferase assay

Firefly luciferase reporter genes containing human GCH promoter region (hGCHpro8.0-Luc, hGCHpro5.2-Luc and hGCHpro0.45-Luc) were constructed as previously described (Suzuki *et al.* 2002a). Sea-pansy luciferase vector, phRG-TK vector (Promega, Madison, WI, USA), was used as an internal control to normalize for variations in transfection efficiency. Cells were transfected by lipofection using LipofectAMINE2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. One day prior to transfection, the cells were plated in 24-well plates and transfected at ~50% confluence with 0.80 µg firefly reporter plasmids and 0.01 µg phRG-TK vector per well. The cells were harvested 48 h after transfection and assayed for firefly and sea-pansy luciferase activities by using a PicaGene Dual Luciferase Assay Kit (Toyooka, Tokyo).

Western blotting

Anti-GCH and anti-sepiapterin reductase (SPR; EC 1.1.1.153) antibodies were described previously (Suzuki *et al.* 1999, 2002a). For preparation of whole-cell extracts for immunoblot analysis, the cell pellet was directly lysed in sodium dodecyl sulfate (SDS)-sample buffer and the supernatant was collected as whole-cell extract. The cell lysate was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (4/20 gradient gel) and transferred to a

polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were visualized with ECL plus (Amersham Biosciences, Little Chalfont, UK).

Statistical analyses

Statistical analyses were performed using the ANOVA Bonferroni's test for multiple comparisons. A level of $p < 0.05$ was accepted as statistically significant. Data represent the mean \pm SEM for each condition.

Results

Attenuation of NGF-induced GCH activity by an MEK inhibitor in PC12D cells

First, we examined the induction of GCH activity by NGF in PC12D cells. GCH activity was significantly increased at 6 h, peaked by 12 h, and remained elevated for at least 48 h after NGF treatment. GCH activity at 12 h after NGF treatment was increased 5-fold compared with that before stimulation (Fig. 1a).

Next, to investigate which is the major intracellular signaling pathway for the induction of GCH activity by NGF, we tested several specific inhibitors for protein kinases known to be activated by NGF. U0126 is a non-competitive MEK inhibitor that prevents the activation of MEK1/2, and is considered to be a more specific MEK inhibitor than PD098059 (Duncia *et al.* 1998; Favata *et al.* 1998). The increase of GCH activity induced by NGF was significantly suppressed by pretreatment with U0126 (Fig. 1b). Inhibitors for PI3K, PKA, p38MAPK and JNK had no apparent effect (Fig. 1b). Cellular BH4 content was also increased by NGF in a U0126-sensitive manner in parallel with GCH activity (data not shown).

U0126-sensitive induction of GCH protein and mRNA by NGF

Western blot analysis revealed that the protein level of GCH was increased by NGF treatment, and that the induction of GCH protein level by NGF was suppressed by pretreatment with U0126 (Fig. 2a). We next determined GCH mRNA levels by the quantitative real-time PCR analysis. As an internal control for normalization, 18S rRNA was also quantified. The amount of GCH mRNA relative to that of 18S rRNA was increased 3–4-fold in PC12D cells after 2-h incubation with NGF (Fig. 2b). The induction of GCH mRNA by NGF was almost abolished by pretreatment with U0126, but not with an inhibitor for p38MAPK (Fig. 2b).

Activation of the human GCH promoter by NGF in PC12D cells

We next examined GCH promoter activity using plasmids containing 8.0, 5.2 and 0.45 kb of the human GCH 5'-flanking region fused to a luciferase reporter gene (hGCHpro8.0-Luc,

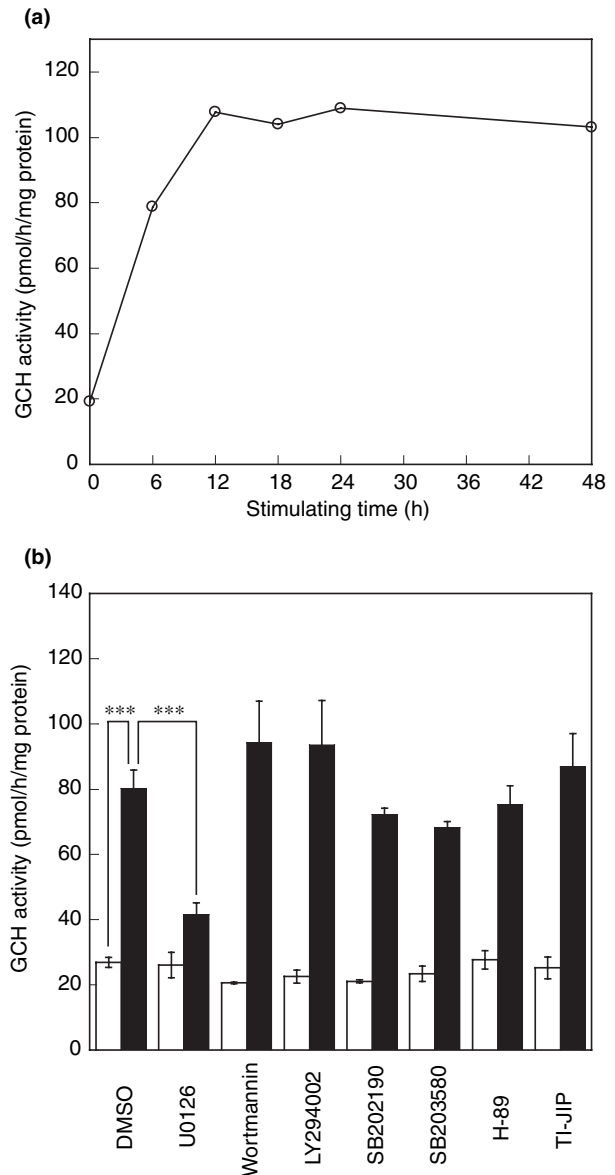


Fig. 1 Increment of GCH activity in response to NGF is attenuated by U0126 in PC12D cells. (a) Time course analysis. Cells were incubated with 50 ng/mL rat recombinant NGF. Data are means from two independent experiments. (b) GCH activity in NGF-stimulated (closed bars) and vehicle-stimulated control (open bars) PC12D cells in the presence of protein kinase inhibitors. Cells were pretreated with 10 μ M U0126, 1 μ M Wortmannin, 1 μ M LY294002, 10 μ M SB202190, 10 μ M SB203580, 30 μ M H-89 or 2 μ M TI-JIP for 30 min, and then incubated with 50 ng/mL NGF for 6 h. Data are means \pm SE values from three independent experiments. *** $p < 0.001$.

hGCHpro5.2-Luc and hGCHpro0.45-Luc, Fig. 3a). As shown in Fig. 3(b), the reporter activity of hGCHpro8.0-Luc was significantly increased in response to NGF in PC12D cells. Deletion of the GCH promoter region up to 453 bp from the transcriptional starting site did not diminish the response to

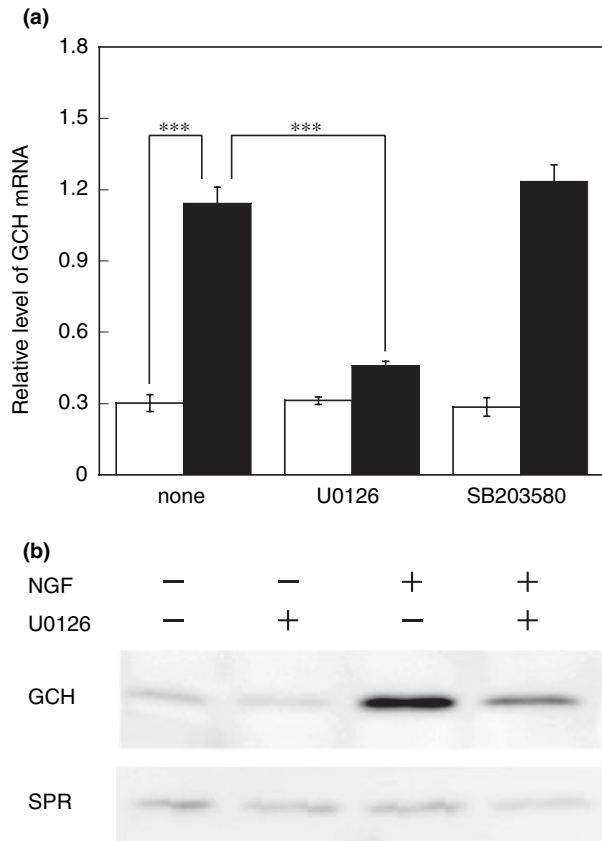


Fig. 2 Induction of GCH mRNA in response to NGF is attenuated by U0126 in PC12D cells. (a) GCH mRNA levels in NGF-stimulated (closed bars) and vehicle-stimulated (open bars) PC12D cells in the presence of protein kinase inhibitors. Levels of the GCH mRNA were expressed as fold activation relative to that in non-stimulated PC12D cells. 18S rRNA was used as the internal control for normalization. Cells were pretreated with 10 μ M U0126, or 10 μ M SB203580, for 30 min, and then incubated with NGF for 2 h. Data are means \pm SE values from three independent experiments. *** p < 0.001. (b) Western blotting was performed by using antibodies against GCH and SPR. Whole-cell extracts were prepared from PC12D cells that had been pretreated with the inhibitors or vehicle only for 30 min and then incubated without or with NGF for 6 h. Whole-cell extracts were separated by SDS-PAGE (4–20% gradient gel) and then analyzed by immunoblotting with antibodies against GCH and SPR.

NGF, suggesting that the 453 bp 5'-flanking region of the GCH gene is required for the NGF-dependent expression. Consistent with the above results (Figs 1 and 2), pretreatment with U0126 completely blocked the NGF-dependent activation of the GCH promoter (Fig. 3b).

Ras- and MEK-dependent activation of the GCH promoter by NGF

The blockage of relative luciferase activity by U0126 suggests that the Ras/MEK pathway might be involved in NGF-dependent GCH promoter activity. To test this possibility further, we examined the effects of MEK and Ras

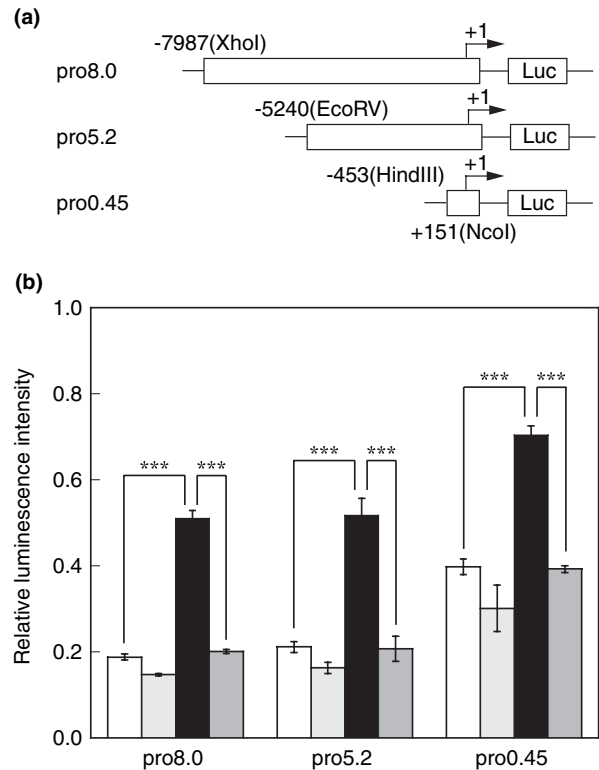


Fig. 3 Induction of the human GCH promoter activity in response to NGF in PC12D cells. (a) Diagram of a reporter plasmid containing 8.0, 5.2 or 0.45 kb of the 5'-flanking region (hGCHpro8.0-Luc, hGCHpro5.2-Luc or hGCHpro0.45-Luc). (b) Relative reporter activity of each plasmid in NGF-stimulated and non-stimulated control PC12D cells was measured. A sea-pansy luciferase vector, pRG-TK, was used as an internal control to normalize for variations in transfection efficiency. The cells were co-transfected with the luciferase reporter vector and the internal control vector 42 h prior to pretreatment with 10 μ M U0126 for 30 min and subsequent treatment with NGF for 6 h. Data were means \pm SE values from three independent experiments. Open bar, none; light gray bar, U0126 only; black bar, NGF only; dark gray bar, U0126 and NGF. *** p < 0.001.

overexpression on the GCH promoter activity. Co-transfection of the PC12D cells with an active form of MEK1 and the wild-type of Ras increased the reporter activity of hGCHpro0.45-Luc by itself (Fig. 4, left). In addition, the dominant-negative form of Ras attenuated GCH promoter activity stimulated by NGF (Fig. 4, right).

Discussion

In the present study, we demonstrated, for the first time, that the Ras/MEK pathway was required for the NGF-mediated transcriptional activation to express the GCH gene in PC12D cells. We previously reported that the Ras/MEK pathway is also required for TH gene expression by NGF. Taken together, our present study suggests that the Ras/MEK pathway is responsible for co-induction of TH and GCH

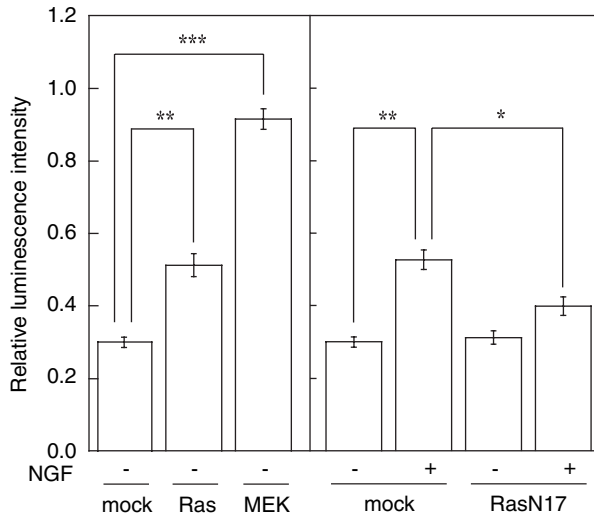


Fig. 4 Effects of Ras and MEK overexpression on the human GCH promoter activity in PC12D cells. PC12D cells co-transfected with pHRG-TK, reporter vector, and 50 ng of the active form of MEK1-, wild-type Ras-, inactive form of Ras-expression vector (MEK1, Ras, or RasN17, respectively), or control vector (pCMV-C, Mock); and the transfected cells were then incubated with or without NGF. Data were means \pm SE values from three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

gene expression. As the induction of both BH4 content and GCH expression by NGF was sensitive to an MEK inhibitor, the Ras/MEK pathway is required for up-regulation of both TH and its essential cofactor, BH4.

Whereas molecular mechanisms and signaling pathways activated by neurotrophins to regulate neurite extension and neural cell survival have been extensively investigated, relatively little is known about those to regulate neurotransmitter-synthesizing enzymes and to determine the neurotransmitter phenotypes. NGF has been reported to up-regulate TH gene expression as well as neurite development and cell survival in peripheral sympathetic and sensory neurons. BH4 is especially essential for TH protein at nerve terminals, because depletion of BH4 content was shown to result in the reduction of both TH immunoreactivity and catecholamine contents at sympathetic nerve terminals in newborn gene-targeted mutant mice of pyruvoyltetrahydropterin synthase, which catalyzes the second step of BH4 biosynthesis (Sumi-Ichinose *et al.* 2001). Our data suggest that the Ras/MEK pathway plays a central role in the up-regulation of CA biosynthesis by the coordinate induction of TH and GCH expression in peripheral catecholaminergic neurons during neural development.

In addition to NGF, other neurotrophins and other neurotrophic factors are known to activate the MEK pathway and to act on catecholaminergic neurons. For instance, BDNF and GDNF are demonstrated to up-regulate TH gene expression in midbrain dopaminergic neurons. Our data

suggest that the Ras/MEK pathway may play an important role in coordinate expression of GCH and TH genes elicited by various stimuli including other neurotrophic factors in central and peripheral catecholaminergic neurons.

Both Ras/MEK and PI3K/Akt pathways are demonstrated to be required for neurite extension and anti-apoptotic effects in PC12 cells. In addition, cooperative action of the Ras/MEK and PI3K/Akt pathways was suggested to up-regulate the gene expression of NMDA receptor 1 and acetylcholine receptors in response to NGF in PC12 cells (Liu *et al.* 2001; Melnikova and Gardner 2001). In contrast, our data suggest, as in the case with TH, that NGF-induced GCH gene expression was dependent on the Ras/MEK pathway, but not on the PI3K/Akt pathway, because PI3K inhibitors wortmannin (Fig. 1b) and LY294002 (data not shown) did not attenuate induction of the GCH activity.

We previously reported that both CRE and AP-1-binding motifs in the proximal region of the TH gene were required for the transcription of the TH gene by NGF via the Ras/MEK pathway in PC12D cells (Suzuki *et al.* 2004). We have demonstrated here that the Ras/MEK pathway also acted on 453 bp of the 5'-flanking region of human GCH promoter in PC12D cells, suggesting that this proximal region is responsible for the transcription of the GCH gene by NGF. This proximal region of the GCH promoter has an SP1/GC box, a non-canonical CRE and a CCAAT box, which are required for basal and cAMP-dependent promoter activities and are conserved among human, rat and mouse GCH genes (Kapatos *et al.* 2000; Hirayama *et al.* 2001). In the context of coordinate induction of TH and GCH, Ras/MEK-dependent CRE-mediated transcription could play an important role in transcriptional regulation of the both genes by NGF.

NGF was shown to cause phosphorylation and transactivation of CREB at least in part through the Ras/MEK-dependent pathway (Ginty *et al.* 1994). CREB was also shown to be required for NGF-dependent survival of sympathetic neurons (Riccio *et al.* 1999). Electrophoretic mobility shift assay demonstrated that CREB bound to the GCH-CRE to a lesser extent than the TH-CRE (Kapatos *et al.* 2000). These reports suggest that CREB is a possible candidate for the Ras/MEK-dependent coordinate regulation of GCH and TH genes by NGF. In addition to CREB, ATF-2 could bind to both the TH- and GCH-CRE. NGF can induce phosphorylation of ATF-2 on the residues critical for transactivation activity in PC12 cells. We recently reported that activated ATF-2 induces the TH gene transcription via CRE (Suzuki *et al.* 2002b). Although Ras/MEK-dependent ATF-2 activation is unclear, ATF-2 is also a candidate for the coordinate induction of TH and GCH genes by NGF.

Alternatively, or concomitantly, it is possible that NGF/Ras/MEK-activated transcription factors required for the expression of the GCH gene are different from those for the TH gene. Whereas the TH-AP-1 binding motif is required for the activation of the TH promoter by NGF, the AP-1

consensus site has not been identified in the GCH promoter region. AP-1 transcription factors such as c-Fos, c-Jun and Egr-1 are well-known immediate early genes induced by NGF in PC12 cells, and have been shown to have a crucial role in the expression of the TH gene (Sukhatme *et al.* 1988; Ginty *et al.* 1994; Ahn *et al.* 1998; Melnikova and Gardner 2001). However, the CCAAT box is characteristic of the GCH gene compared with the TH genes. C/EBP β , which can bind to the GCH-CCAAT box (Kapatos *et al.* 2000), was demonstrated to be activated by NGF in an MEK-dependent manner (Sterneck and Johnson 1998).

In sympathetic neurons innervating mouse sweat gland in the footpad, suppression of GCH expression and BH4 content, but not TH expression, was observed during developmental change in neurotransmitter phenotype noradrenergic to cholinergic, suggesting that GCH expression can be controlled independently of TH expression (Habecker *et al.* 2002). Extracts from sweat gland-containing footpads were reported to suppress BH4 content in cultured sympathetic neurons (Habecker *et al.* 2002). NGF was shown to regulate the innervation of sympathetic neurons in the sweat gland. These findings, including our present study, suggest that NGF/Ras/MEK-dependent co-induction of TH and GCH gene expression in these neurons may be modulated by unidentified factors in the extract from the sweat gland. The possible difference of transcription factors required for the TH and GCH expressions by NGF may underlie the modification of the GCH expression independent of the TH expression.

In mice carrying a TH transgene, the change in TH protein level and enzymatic activity was very small in spite of a great increase in TH mRNA level (Kaneda *et al.* 1991). Our previous study demonstrated that TH promoter activity was highly elevated by NGF in PC12D cells, whereas the increase of TH mRNA was small, and then the TH activity was unchanged (Suzuki *et al.* 2004). In contrast, our present study demonstrated that GCH enzyme activity was enhanced by NGF to a similar extent as GCH mRNA level, suggesting that GCH activity is directly regulated by transcriptional activation of the GCH gene. These reports suggest that some unknown additional mechanisms at post-transcription level may exist to regulate TH enzyme activity independent of GCH activity.

Co-induction of GCH and NOS by cytokines has been reported in endothelial cells and leucocytes. The Ras/MEK pathway is also required for the induction of nNOS, which needs BH4 as a cofactor, in NGF-treated PC12 cells (Schonhoff *et al.* 2001). It is also known that the expression of all three NOS isozymes can be regulated by the Ras/MEK pathway in various cell types (Chen *et al.* 1999; Zheng *et al.* 1999; Schonhoff *et al.* 2001). Based on these reports, our data also suggest that the Ras/MEK pathway might be involved in the coordinate induction of the GCH and NOS expressions in neural and non-neural cells.

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