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 (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.

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; Szymey 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 (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 (Katoh-Semba 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 (Katoh-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'-GACCACCCCTGGCCATTTGG-3' (forward) and 5'-TTCCACATCTTGCCATTTGG-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 (hGCHpro0.8-Luc, hGCHpro0.8-Luc and hGCHpro0.45-Luc) were constructed as previously described (Suzuki et al. 2002a). Sea-pansy luciferase vector, phiRG-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 phiRG-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 (Toyoink, 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 ± 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, 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...
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 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 hGCH-pro8.0-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.
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 PC12 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 PC12 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/EBPβ, 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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References


