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Regulation of neuropathy target esterase by the cAMP/protein kinase A signal

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ABSTRACT

As a phospholipase B, neuropathy target esterase (NTE) is responsible for the conversion of phosphatidylcholine (PC) to glycerophosphocholine (GPC). We examined the role of cAMP in the regulation of NTE in mammalian cells. Endogenous NTE activity was increased by cAMP-elevating chemicals, including dibutyryl cAMP, forskolin and forskolin plus 1-isobutyl-3-methylxanthine (IBMX), but decreased by the adenyl cyclase inhibitor SQ22536 which can reduce intracellular cAMP levels. Exogenous GFP-tagged NTE activity was not affected by changes in intracellular cAMP. NTE protein levels were up-regulated by the cAMP-elevating reagents and down-regulated by the inhibitor. The effect of the adenyl cyclase activator forskolin on NTE protein and mRNA levels was blocked by pretreatment with the protein kinase A (PKA) activity inhibitor H89. In addition, we found that changes in GPC, but not PC, levels were correlated with cAMP induced changes in NTE activity. These results are the first evidence that cAMP/PKA signals regulate NTE expression and GPC content in mammalian cells.

1. Introduction

Neuropathy target esterase (NTE) was identified 40 years ago as the primary target of the organophosphorus compounds (OP) that cause OP-induced delayed neuropathy (OPIDN), a syndrome characterized by paralysis of the lower limbs due to degeneration of long axons in the spinal cord and peripheral nerves. It has been suggested that the inhibition and subsequent ageing of NTE initiates OPIDN [1]. NTE has been found to be a serine esterase protein that is highly conserved among a diverse range of animal taxa, including insects, nematodes, yeast and bacteria [2]. NTE is present not only in neurons but also in a variety of non-neural tissues, including those found in the intestines, placenta, lymphocytes, kidneys, liver and testicles [3,4]. NTE is a polypeptide of 1, 327 amino acids anchored to the cytoplasmic face of the endoplasmic reticulum (ER) through an amino-terminal transmembrane segment in neurons and some non-neural cells [5,6].

Phosphatidylcholine (PC) is a major class of glycerophospholipids present in all mammals that plays a critical role in membrane structure and cellular signaling. A previous study determined that NTE displayed potent lysophospholipase activity in the mouse brain [7]. NTE has been further characterized as a phospholipase B responsible for the conversion of PC to glycerophosphocholine (GPC) and regulating PC homeostasis in *Drosophila* and the mouse [8–10].

Although some of NTE's characteristics have been elucidated, little is known about the regulation of its activity and expression. Previous studies have shown that the N-terminal regulatory part of NTE contains three putative cyclic AMP (cAMP)-binding sites that could function as a putative regulatory domain; evidence that NTE could be regulated by cAMP [2,11]. Although Dremier et al. were unable to demonstrate that radioactively-labeled cAMP bound to the regulatory domain of NTE in purified recombinant *E. coli* [12], the possibility that cAMP binds to NTE in mammalian cells cannot be ruled out. It is also possible that lowering intracellular cAMP levels may affect NTE activity. In a previous study, we demonstrated that NTE can interact with the protein Gβ2 through its catalytic domain, and that pertussis toxin, a G protein signal pathway influencing factor, and the depletion of Gβ2 by RNA interference, down-regulated NTE activity [13]. These results indicate that the regulation of NTE catalytic activity by both pertussis toxin treatment and knockdown of Gβ2 expression may be associated with change in cAMP levels. In this paper we fur-

Abbreviations: cAMP, cyclic AMP; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; FSK, forskolin; GPC, glycerophosphocholine; IBMX, 1-isobutyl-3-methylxanthine; NTE, neuropathy target esterase; OPIDN, organophosphorous compound-induced delayed neuropathy; PC, phosphatidylcholine; PKA, protein kinase A.

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ther investigate potential pathways for the regulation of NTE by cAMP.

2. Materials and methods

2.1. Cells and chemicals

HeLa cells were purchased from the Cell Center of the Chinese Academy of Medical Sciences (Beijing, China). The monkey kidney-derived cell line COS7 was kindly provided by Prof. Y. L. Wang, State Key Laboratory of Reproductive Biology (Beijing, China). Cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA). NTE-GFP plasmid constructs was kindly provided by Dr Y. Li (MRC Toxicology Unit, UK). Rabbit anti-NTE polyclonal antibody (H-200), rabbit anti-actin monoclonal antibody (C-2) and goat anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Paraoxon, dibutyryl cAMP (dbcAMP), 1-isobutyl-3-methylxanthine (IBMX), forskolin (FSK), 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536) and N-[2-(p-bromocinnamy-lamino)ethyl]-5-isoquino-linesulfona-mide (H89) were purchased from Sigma (St. Louis, MO, USA). Mipafox and phenyl valerate were synthesized in our laboratory. The enhanced chemiluminescence (ECL) reagent was purchased from Pierce Biotechnology (Rockford, IL, USA). The Trizol reagent and transfection reagent Lipofectamine 2000 were purchased from Invitrogen Life Technologies (Groningen, the Netherlands). First-strand cDNA synthesis kit (ReverTra Ace-α-^{TM}) for the reverse transcription-polymerase chain reaction (RT-PCR) was obtained from Toyobo Co. Ltd. (Osaka, Japan). The cAMP radioimmunoassay kit was made by the Sun Biomedical Technology Co. Ltd (Beijing, China).

2.2. Cell culture and transfection

Cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in the logarithmic phase of growth and sub-cultured at 3–4-day intervals. Transient transfection of NTE-GFP in COS7 cells was performed using Lipofectamine 2000 according to the manufacturer's protocol.

2.3. Cell treatment

To determine the effect of cAMP on the regulation of NTE, HeLa cells and transfected COS7 cells, both plated at a density of 1×10^6 cells per 100-mm culture dish, were incubated with either a membrane permeable analog of cAMP (200 μM dbcAMP), an adenyl cyclase activator (10 μM forskolin), a general inhibitor of phosphodiesterases (10 μM forskolin plus 100 μM IBMX), or an adenyl cyclase inhibitor (50 μM SQ22536), for 24 h. To observe the effect of PKA on the regulation of NTE some HeLa cells (1×10^6/dish) were also incubated for 24 h with 10 μM H89, a PKA activity inhibitor, in the presence or absence of forskolin. Apart from dbcAMP which was dissolved in redistilled H_2O, all other chemicals were dissolved in DMSO.

2.4. Western blotting analysis

The cells were trypsinized, washed twice with ice-cold PBS and homogenized in 1 ml TE buffer (50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0) on ice. The homogenate was then centrifuged at $100 \times g$ at 4 °C for 2 min after which the supernatant was centrifuged at $100{,}000 \times g$ at 4 °C for 45 min. The deposit was dissolved in TN buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris–HCl, pH 8.0) and the protein concentration determined according to the method of Lowry et al. using bovine serum albumin (BSA) as a standard [14]. The protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis with a 5% stacking gel and 8% separating gel, and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corporate, Billerica, MA, USA). Following transfer, membranes were blocked with 1× Tris-buffered saline (TBS) buffer containing 0.05% Tween-20 and 5% non-fat milk for at least 1 h at room temperature, then incubated with rabbit polyclonal anti-NTE antibody (diluted 1:1000) before finally being incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:5000). Immunoreactive bands were detected using a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA) and standard ECL reagents. The blot was stripped and re-probed with anti-actin antibody to detect the loaded protein.

2.5. RT-PCR analysis

Total RNA was isolated from HeLa cells with Trizol reagent according to the manufacturer's instructions and quantified spectrophotometrically after DNase I treatment. The total RNA (1 μg) was reverse transcribed using a first-strand cDNA synthesis kit in a final volume of 20 μl according to the manufacturer's instructions. PCR analyses were then performed on aliquots of the cDNA preparations to detect NTE and GAPDH gene expression with a thermal cycler (Eppendorf AG 22331 MasterCycler, Hamburg, Germany). The reactions were carried out in a volume of 25 μl containing

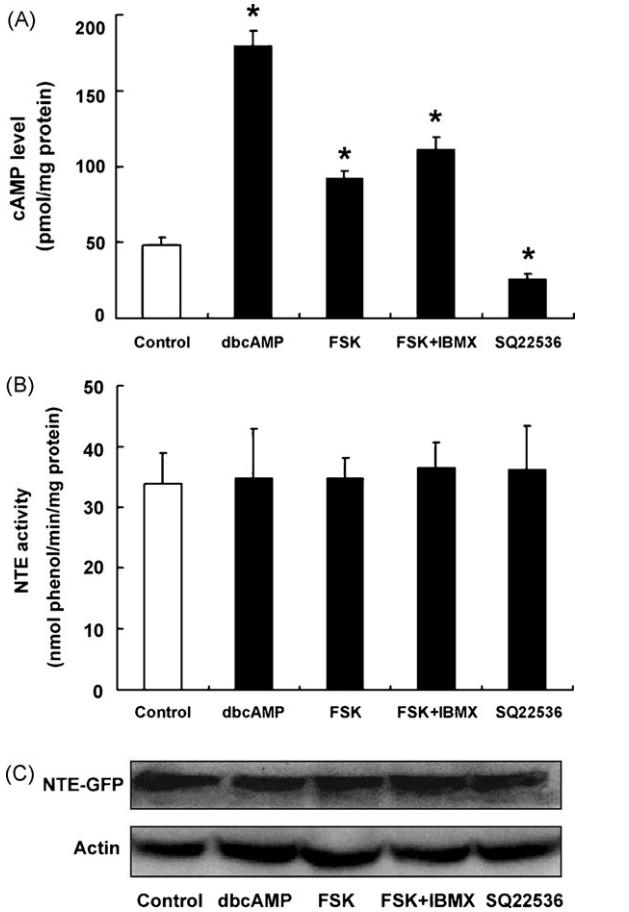

Fig. 1. cAMP level (A), enzyme activity (B) and protein level (C) of NTE in COS7 cells transfected with NTE-GFP and treated with either 200 μM dbcAMP, 10 μM forskolin, 10 μM forskolin (FSK) plus 100 μM IBMX or 50 μM SQ22536. See Section 2 for details. The experiment was done in triplicate and repeated three times. Data were analyzed by one-way ANOVA. *$P < 0.05$.