CREB Is Required for cAMP/PKA Signals Upregulating Neuropathy Target Esterase Expression

Jia-Xiang Chen^{1–3} and Yi-Jun Wu¹

Neuropathy target esterase (NTE), which has been proposed as the primary target of organophosphorus compounds that cause delayed neuropathy with degeneration of nerve axons, is expressed primarily in neural cells but is also detected in non-neural cells. However, little is known about the regulation of NTE gene in cells. We found that a cyclic-AMP (cAMP)-response element (CRE) exists in the 5' flanking sequence of NTE gene in HeLa cells, which implies that NTE may be regulated by the transcription factor cAMP-response element-binding protein (CREB). In the study, knockdown of CREB decreased the protein and mRNA levels of NTE and inhibited the upregulation by cAMP/PKA signaling. Moreover, we observed that knockdown of CREB significantly decreased luciferase activity of the NTE gene promoter, while it had no effect on that of the CREB binding sites of mutated NTE gene promoter and truncated NTE gene promoter lacking the CREB binding site. cAMP/PKA signals could increase NTE reporter gene activity, while knockdown of CREB inhibited the increase. We found that the transcription factor CREB can bind to the promoter sequence of NTE by chromatin immunoprecipitation. In conclusion, we provided evidence that CREB is required for cAMP/PKA signals upregulating NTE expression in HeLa cells.

Introduction

NEUROPATHY TARGET ESTERASE (NTE) was proposed over 40 years ago as the primary target of organophosphorus compounds (OP) that cause an effect, OP-induced delayed neuropathy (OPIDN), which is characterized by a paralysis of the lower limbs due to degeneration of long axons in the spinal cord and in peripheral nerves. The inhibition and subsequent aging of NTE has been proposed to be an initiating event in OPIDN (Johnson, 1974, 1977; Hou *et al.*, 2008). NTE is a polypeptide of 1327 amino acids and is anchored to the cytoplasmic face of endoplasmic reticulum through an amino-terminal transmembrane segment in neurons and some non-neural cells (Li *et al.*, 2003; Akassoglou *et al.*, 2004). NTE has been proved to be highly conserved among species including insects, nematodes, yeast, and bacteria (Lush *et al.*, 1998; Chang and Wu, 2009, 2010).

Previous studies showed that NTE displayed potent lysophospholipase activity in the mouse brain. This activity has been characterized as a novel phospholipase B responsible for the conversion of phosphatidylcholine (PC) to glycerophosphocholine (GPC) and regulating PC homeostasis in *Drosophila* and the mouse (Zaccheo *et al.*, 2004; Muhlig-Versen *et al.*, 2005; Read *et al.*, 2009). In mice, complete inactivation of the NTE gene led to embryonic lethality re-

sulting from placental failure and impaired vasculogenesis (Winrow *et al.* 2003; Moser *et al.* 2004). In adult *Drosophila*, loss of Swiss cheese/NTE activity causes neuronal and glial death (Akassoglou *et al.* 2004; Muhlig-Versen *et al.* 2005). Taken together, these studies suggest that NTE is essential for embryonic and nervous development.

Elucidation of the role played by NTE in OPIDN and neural development is of obvious neurobiological interest. However, NTE is present not only in neurons but also in non-neural tissues including those found in the intestines, placenta, lymphocytes, kidneys, liver, and testicles (Williams, 1983; Li *et al.*, 2003; Winrow *et al.*, 2003; Chen *et al.*, 2012). Therefore, NTE may play a universal role in mammalian neurons and non-neural cells. Further, there is a putative homologue in *Saccharomyces cerevisiae* (Lush *et al.*, 1998), indicating that NTE might be involved in a common physiological process from yeast to mammal (Li *et al.*, 2003).

Although the characteristics of NTE have been elucidated in some animals, little is known about the regulation of NTE. In a previous study, we demonstrated that NTE can interact with the protein Gβ2 through its catalytic domain. We also showed that pertussis toxin, a G protein signal pathway influencing factor, and the depletion of Gβ2 by RNA interference, can downregulate NTE activity *in vivo* (Chen *et al.*, 2007). NTE expression can also be regulated by cyclic-AMP

¹Laboratory of Molecular Toxicology, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, People's Republic of China.

²Graduate University of Chinese Academy of Sciences, Beijing, People's Republic of China.

³Medical School of Nanchang University, Nanchang, People's Republic of China.

(cAMP)/PKA signal in HeLa cells (Chen *et al.*, 2010). The TFSEARCH software was used to identify putative consensus binding sites for cAMP-response element-binding protein (CREB) on the NTE gene promoter. NTE proximal promoter contains a cAMP-responsive element (CRE) between -83 and -76 base pairs (bp). However, there is no existing evidence whether the NTE expression can be regulated by CREB in mammalian cells.

The aim of the present study is to investigate whether cAMP/PKA/CREB signals regulate NTE expression in mammalian cells. This finding will facilitate the future investigation of the molecular mechanism governing the human NTE gene expression.

Materials and Methods

Cells and chemicals

HeLa cells were purchased from the Cell Center of the Chinese Academy of Medical Sciences (Beijing, China). Cell culture reagents were obtained from Gibco BRL. Rabbit anti-NTE polyclonal antibody (H-200), rabbit anti-actin monoclonal antibody (C-2), Rabbit anti-CREB1 polyclonal antibody (sc-58), and goat anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology. Forskolin and N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) were obtained from Sigma. The pGenesil-1 vector was obtained from Genesil Biotechnology Co., Ltd. The enhanced chemiluminescence (ECL) reagent was obtained from Pierce Biotechnology. The TRIzol reagent, transfection reagent Lipofectamine 2000, and chromatin immunoprecipitation (ChIP) system were purchased from Invitrogen Life Technologies. First-strand cDNA Synthesis Kit (ReverTra Ace- α -TM) for reverse transcription-polymerase chain reaction (RT-PCR) was obtained from Toyobo Co. Ltd. EX Taq Hot Start DNA polymerase was purchased from Takara. Site-Directed Mutagenesis Kit was obtained from TransGen. Dual-Luciferase® Reporter Assay System was purchased from Promega.

Cell culture and transfection

Cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. Incubations were carried out at 37°C in a humidified atmosphere of 5% CO₂/95% air. The cells were maintained in the logarithmic phase of growth and subcultured at 3–4 days intervals. Transient transfection of control shRNA and RNAi-CREB in HeLa cells was performed using Lipofectamine 2000 according to the manufacturer's protocol.

Construction of CREB shRNA vector

To construct shRNA vector for human CREB (NM_004379), the pGenesil-1 vector (Genesil Biotechnology Co., Ltd.) was digested by *Bam*HI and *Hind*III. Next, CREB-F: 5'-GATCCCCCATCATCTGCTCCACCGTTTCAAG AGAAC GGTGGGAGCAGATGATGTTTTGGAAA-3' and CREB-R: 5'-AGCTT TTCAAAAACATCATCTGCTCCACCGTTCT CTTGAAACGGTGGGAGCAGATGATGGGG-3' (wherein the siRNA sequence for CREB was shown in bold and restriction sites were underlined) were annealed to form a double-stranded DNA with *Bam*HI and *Hind*III sites at the 5' and 3' ends, respectively. The double-stranded DNA was li-

gated into the *Bam*HI and *Hind*III sites of the pGenesil-1 vector. The forward primer of RNA interference control construct (control shRNA) was 5'-GATCCCCCACTTACTCCGTCAC CTCGTTCAAGAGACGAGGTGACGGAGTAAGTGTGTTTT GGAAA-3' and the reverse primer was 5'-AGCTTTTCCA AAAAC ACTTACTCCGTCACCTCGTCTCTTGAACGAGG TGACGGAGTAAGTGGGG-3'. All the constructs were verified by DNA sequencing.

Cell treatment

HeLa cells were transiently transfected with control shRNA and RNAi-CREB for 24 h; the cells were treated with forskolin (10 μ M), H89 (10 μ M), an inhibitor of PKA activity, and H89 plus forskolin for 24 h to observe the effect of cAMP/PKA/CREB on the mRNA and protein levels of NTE.

Western blotting analysis

The cells were trypsinized, washed twice with ice-cold PBS, and homogenized in 1 mL TE buffer (50 mM Tris-HCl and 0.2 mM EDTA, pH 8.0) on ice. The homogenate was then centrifuged at 100 g at 4°C for 2 min after which the supernatant was then centrifuged at 100,000 g at 4°C for 45 min. The deposit was dissolved in TN buffer (150 mM NaCl, 1% Triton X-100, and 50 mM Tris-HCl, pH 8.0) and the protein concentration determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 5% stacking gel and 8% separating gel and transferred to polyvinylidene fluoride membrane (Millipore Corporation). Following transfer, membranes were blocked with 1× Tris-buffered saline buffer containing 0.05% Tween-20 and 5% nonfat milk for at least 1 h at room temperature, then incubated with rabbit polyclonal anti-NTE antibody (diluted 1:1000), and finally incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1: 5000). Immunoreactive bands were detected using a ChemiDoc XRS system (Bio-Rad) and standard ECL reagents. The blot was stripped and re-probed with anti-actin antibody to detect the loaded protein.

RT-PCR analysis

Total RNA was isolated from HeLa cells with TRIzol reagent according to the manufacturer's instructions and spectrophotometrically quantified after DNase I treatment. The total RNA (1 μ g) was reverse transcribed using the first-strand cDNA synthesis kit in a final volume of 20 μ L, according to the manufacturer's instructions. PCR analyses were then performed on the aliquots of the cDNA preparations to detect NTE and GAPDH gene expression using a thermal cycler (Eppendorf AG 22331 MasterCycler). The reactions were carried out in a volume of 25 μ L containing 2.5 U of Taq DNA polymerase, 0.2 mM deoxyribonucleoside triphosphates, 1× reaction buffer, and 100 pmol of specific 5' and 3' primers. Specific primers were designed for the NTE sequence with Primer Express software (Applied Biosystems). The forward primer was 5'-CCAAGAGTTCGGCT GTCA-3' and the reverse primer was 5'-CACAATGAGGA TGCAGTCGG-3'. The forward and reverse primers for CREB sequence were 5'-CACCAGGAGTGCCAAGGATT-3' and 5'-CTGCTGCATTGGTCATGGTT-3'. The forward and reverse

primers for *GAPDH* sequence were 5'-ATGCTGGCGCTGAGTACGTC-3' and 5'-GGTCATGAGTCCTTCCACGATA-3', respectively. For the indicated gene amplification, the denaturation cycle (94°C, 5 min) was followed by 35 cycles, each cycle consisting of DNA denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s, and elongation at 72°C for 1 min, and an elongation cycle at 72°C for 10 min at the end of amplification. PCR products were detected with a ChemiDoc XRS (Bio-Rad) using 1% agarose gel electrophoresis and ethidium bromide staining.

Cloning of the human NTE promoter and site-directed mutagenesis

A 1756 bp long DNA sequence comprising the 5' flanking sequence upstream of the transcription start site of the human NTE gene was amplified by PCR using the following oligonucleotides: 5'-CGGAGCTCCCTGACTGCCGTGGACCGTAGGCCTGGACTGCAGAGACCCCGCCCCGAC-3' and 5'-CTAAGCTTATTCCAGCTGGACGGCCGATCTGGTAGCCCGGAGTCTGGGAAATCAAGGAGT-3', which was flanked by *SacI* and *HindIII* restriction sites (restriction sites were underlined). The amplicons were gel-purified and cloned into the promoterless luciferase reporter plasmid pGL3-basic (Promega). The resulting construct was designated pGL3-NTE promoter. The pGL3-NTE promoter constructs were confirmed by sequencing prior to use in transient transfection experiments.

The constructs containing mutated CREB binding sites were obtained using the Site-Directed Mutagenesis Kit (TransGen) following the manufacturer's instructions. The nucleotide sequence TGACGACT in the CRE binding site, at -83 to -76 bases in the 5' flanking region of NTE gene, was mutated to TGAATACT. The forward primer was 5'-AGGTGGGCCCCA GATTGAATACTTGCACGGGT-3' and the reverse primer was 5'-ATTCAATCTGGGCCACCTCTCCCTGCC-3'. The construct was designated pGL3-Mut-NTE promoter. The mutagenesis was performed with pGL3-NTE promoter construct as a template. The successful mutants were confirmed by sequence.

The constructs without CREB binding site were also constructed to pGL3-basic (was designated pGL3-NTE-51 promoter), the forward and reverse primers of the truncated NTE gene promoter were 5'-ATTTCCCAGGACTCCGGGCTACCAGATCGGCCGTCCAGCTGGAATCAACCG-3' and 5'-AGCTCGGTTGATTCCAGCTGGACGGCCGATCTGGTAGCCCGGAGTCTGGGAAATAGCT-3'.

Reporter gene analysis

To observe whether CREB was required for the NTE promoter gene activity, HeLa cells were transiently transfected with control siRNA or RNAi-CREB, then the different firefly luciferase constructs, such as pGL3-NTE-51 promoter, pGL3-NTE promoter or pGL3-Mut-NTE promoter, and Renilla luciferase phRL-TK vector (Promega) were transfected to determine the transcriptional activity of the NTE reporter gene.

To determine the effect of cAMP/PKA signal on the NTE promoter gene activity, HeLa cells were transiently transfected with pGL3-NTE promoter. Twenty-four hours after transfection, the cells were treated with forskolin (10 μ M), H89 (10 μ M), or H89 plus forskolin for 24 h. The transcrip-

tional activity of the NTE reporter gene was determined using a dual luciferase assay kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity (phRL-TK) and expressed as fold change over the mean of the untreated control group.

Chromatin immunoprecipitation

The ChIP experiment was performed following a previously described protocol (Dammer *et al.*, 2007). Briefly, HeLa cells were cross-linked with 1% formaldehyde for 10 min, followed by termination of the cross-linking with 0.125 M glycine. Cell lysates were prepared and chromosomal DNA was sonicated to reach average sizes between 200–1000 bp. The lysate was immunoprecipitated with either rabbit IgG or an anti-CREB antibody. Upon reversal of the cross-linking, the purified DNA fragments were PCR-amplified for the promoter regions of NTE, GAPDH, and NR4A2 gene. The forward and reverse primers for NTE gene sequence were 5'-TTTGCTCCATCCCTAGTCC-3' and 5'-AAGGAGTGAAGGAACCCGT-3'; the primers for GAPDH gene were 5'-GAGAAAGTAGGGCCCGGCTA-3' and 5'-GGTCTTGA GGCTGAGCTACG-3'; the primers for NR4A2 gene were 5'-CCACCCAAGCTGGC TACCAA-3' and 5'-GTTTATGTGGCTTGCGCTGC-3'. Output (immunoprecipitated promoter region) was normalized to input DNA.

Statistical analysis

Values were expressed as the mean $\pm$ SEM. For comparison of the two groups, the statistical difference was determined by Student's *t*-test. Other data were evaluated by one-way analysis of variance with Newman-Keuls Multiple range test. For each test, *p*-values less than 0.05 (*p* < 0.05) were considered statistically significant.

Results

CREB is required for NTE expression

To investigate whether CREB was required for NTE expression, the control shRNA and RNAi-CREB constructs were transiently transfected to observe NTE expression in HeLa cells. As shown in Figure 1, the protein and mRNA levels of CREB were significantly decreased after transfection with RNAi-CREB, indicating that the constructed RNAi-CREB vector works well. The protein and mRNA levels of NTE were significantly decreased in the HeLa cells transfected with RNAi-CREB compared with the control cells, indicating that CREB is required for NTE expression.

CREB binds to NTE promoter in vivo

Next, we wished to determine whether the CREB protein, a well characterized transcription factor, could bind to the CRE site in the NTE gene promoter. ChIP assay using CREB-specific antibody revealed that CREB could indeed bind to the NTE gene promoter (Fig. 2).

CREB regulates NTE reporter gene activity

To further define the mechanism by which CREB modulates NTE mRNA expression, we constructed reporter constructs, such as a pGL3-NTE-51 promoter without CREB binding site, a pGL3-NTE promoter having CREB binding

FIG. 1. cAMP-response element-binding protein (CREB) knockdown downregulates expression of neuropathy target esterase (NTE) protein and mRNA. Cells were cultured at a density of 1×10^6 cells per 100-mm culture dish and were transiently transfected with control shRNA and RNAi-CREB for 48 h, respectively. **(A)** The NTE and CREB protein levels were determined by Western blotting analysis; actin was used as a loading control. **(B)** The relative protein levels of NTE and CREB presented in **(A)** were quantified by densitometry. **(C)** The NTE and CREB mRNA levels were determined by the semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), GAPDH was used as a loading control. **(D)** The relative mRNA levels of NTE and CREB presented in **(C)** was quantified by densitometry. The experiments were done in triplicate and repeated three times. Data were analyzed by Student's *t*-test. The results are presented as Mean $\pm$ SEM, $n=3$, $*p<0.05$.

site, and a pGL3-Mut-NTE promoter containing mutated CREB binding site and transiently transfected HeLa cells. The cells were co-transfected with these reporter constructs and control shRNA or RNAi-CREB for 48 h. As shown in Figure 3, the luciferase activity of pGL3-NTE-51 promoter

was significantly lower than that of pGL3-Mut-NTE promoter and pGL3-NTE promoter, which might be due to pGL3-NTE-51 promoter having shorter bases and less transcription factors than pGL3-Mut-NTE promoter or pGL3-NTE promoter. However, knockdown of CREB significantly decreased luciferase activity of pGL3-NTE promoter, while it had no effect on that of pGL3-Mut-NTE promoter and pGL3-NTE-51 promoter, indicating that CREB could upregulate transcriptional activity of NTE gene.

cAMP/PKA signals is involved in upregulation of NTE by CREB

To determine whether cAMP/PKA signals could increase NTE reporter gene activity, we transiently transfected the cells with pGL3-NTE promoter in the absence or presence of knockdown of CREB, then treated the cells with FSK, H89, and H89 plus FSK for 24 h. As shown in Figure 4, the luciferase activity of pGL3-NTE promoter was significantly decreased after treatment with H89 and increased with FSK treatment, while FSK could not abrogate the decrease. Upon knockdown of CREB, however, H89, FSK, and H89 plus FSK had no effect on the luciferase activity.

To further assess whether cAMP/PKA signals are involved in the upregulation of NTE by CREB, HeLa cells were transfected with control shRNA and RNAi-CREB, respectively, and then were treated with H89, FSK, and H89 plus FSK for 24 h. As shown in Figure 5, the protein and mRNA

FIG. 2. Binding of CREB to the NTE promoter *in vivo*. Sheared chromatin prepared from HeLa cells was immunoprecipitated (IP) with IgG or anti-CREB antibody and the bound DNA was isolated according to the manufacturer's instructions (Biotime). The immunoprecipitated DNA was used as a template for PCR using the designed primer sets. Immunoprecipitate of GAPDH and NR4A2 promoter (Conkright *et al.*, 2003) were negative and positive control, respectively.

FIG. 3. CREB is involved in the regulation of NTE promoter luciferase activity. HeLa cells were transfected with reporter gene plasmids such as pGL3-basic, pGL3-NTE-51 promoter, pGL3-NTE promoter, and pGL3-Mut-NTE promoter in the presence or absence of RNAi-CREB. 48 h after transfection, luciferase activity in the cell lysates was determined by luminometry. The experiments were done in triplicate and repeated three times. Data were analyzed by Student's *t*-test. The results are presented as Mean $\pm$ SEM, $n=3$, * $p<0.05$.

levels of NTE were significantly decreased after treatment with H89 and increased with FSK treatment, while FSK could not abrogate the decrease. Upon inhibition of CREB, however, H89, FSK and H89 plus FSK had no effect on the NTE protein and mRNA level.

Discussion

In the present study, we provided evidence for the first time that cAMP/PKA/CREB signals could upregulate the protein expression of NTE in HeLa cells.

NTE was originally identified in adult vertebrate neural tissue as a protein targeted by OP, which cause the syndrome OPIDN, characterized by axonal degeneration (Johnson, 1974). However, NTE is present not only in neurons but also in many non-neural tissues such as intestine, placenta, lymphocytes, kidneys, liver, and testicles (Williams, 1983; Li *et al.*, 2003; Winrow *et al.*, 2003; Chen *et al.*, 2012). In addition,

NTE has shown to catalyze hydrolysis of PC to GPC (Zaccheo *et al.*, 2004), indicating that NTE may play an important role in maintaining cellular phospholipid homeostasis.

CREB is a leucine zipper-containing transcription factor that regulates the expression of several genes by binding to CRE sequences in the promoter of the target gene (Mayr and Montminy, 2001; Sands and Palmer, 2008; Yamamizu *et al.*, 2012; Wu *et al.*, 2012).

CREB is activated by diverse stimuli such as growth factors, hormones, and cytokines and plays important roles in various cellular processes, including proliferation, survival, development, and adaptive responses (Rudolph *et al.*, 1998; Manta-madiotis *et al.*, 2012; Rexach *et al.*, 2012). We found that there was a putative consensus binding site for CREB in the NTE promoter, indicating that NTE might be regulated by CREB. In this study, knockdown of CREB significantly decreased the protein and mRNA levels of NTE in HeLa cells about 70%. CREB is the common target of cAMP/PKA signaling (Impey *et al.*, 2004; Zhang *et al.*, 2005); PKA can phosphorylate CREB at Ser 133, a post-translational modification that is essential for its transcriptional activity (Gonzalez and Montminy, 1989; Mayr and Montminy, 2001). We also found that NTE expression was upregulated by cAMP/PKA signal (Chen *et al.*, 2010). In the present study, forskolin and H89 significantly increased and decreased NTE expression without knockdown of CREB in the HeLa cells. Upon CREB knockdown, both forskolin and H89 had no effect on the expression of NTE, indicating that CREB was required for regulation of NTE by cAMP/PKA signal.

To investigate whether cAMP/PKA/CREB affects transcription activity of NTE gene, we constructed an NTE gene promoter, a CREB binding site mutated NTE gene promoter, and a truncated NTE gene promoter lacking the CREB binding site into pGL3-basic vector and transfected to HeLa cells. We found that knockdown of CREB significantly decreased luciferase activity of NTE gene promoter about 50%, while it had no effect on the CREB binding site of mutated NTE gene promoter and truncated NTE gene promoter lacking the CREB binding site. The difference in the decrease between the protein and mRNA levels, and luciferase activity of NTE after knockdown of CREB might be due to the difference of the assay methods.

Although the CREB binding site in the pGL3-Mut-NTE promoter was mutated, it might have other transcription

FIG. 4. cAMP/PKA/CREB signal is involved in the regulation of NTE promoter luciferase activity. HeLa cells were transfected with reporter gene plasmid pGL3-NTE promoter. Twenty four hours after transfection, cells were treated with FSK, H89, and H89 plus FSK for 24 h and luciferase activity in the cell lysates was quantified by luminometry. The experiments were done in triplicate and repeated three times. Data were analyzed by one-way analysis of variance (ANOVA). The results are presented as Mean $\pm$ SEM, $n=3$, * $p<0.05$.

FIG. 5. cAMP/PKA/CREB signal is required for the regulation of NTE expression. Cells were cultured at a density of 1×10^6 cells per 100-mm culture dish and were transiently transfected with control shRNA and RNAi-CREB for 24 h, respectively; the cells were then treated with H89 alone or H89 plus forskolin. **(A)** The NTE protein level was determined by Western blotting analysis, Actin was used as a loading control. **(B)** The relative level of NTE protein presented in **(A)** was quantified by densitometry. **(C)** The NTE mRNA level was determined by the semiquantitative RT-PCR, GAPDH was used as a loading control. **(D)** The relative level of NTE mRNA presented in **(C)** was quantified by densitometry. The experiments were done in triplicate and repeated three times. Data were analyzed by one-way ANOVA. The results are presented as Mean $\pm$ SEM, $n = 3$, $*p < 0.05$.

factors that might regulate *NTE* transcription. Thus, the luciferase activity of pGL3-Mut-NTE promoter and pGL3-NTE promoter significantly increases more than that of pGL3-NTE-51 promoter. cAMP/PKA signals increased *NTE* reporter gene activity, while knockdown of CREB inhibited the increase. The results indicated that cAMP/PKA/CREB could upregulate transcriptional activity of *NTE* gene. We also identified that transcription factor CREB can bind to the promoter sequence of *NTE* by ChIP.

It was found that diisopropylphosphorofluoridate, an OPIDN-causing compound, caused a decrease in the levels of PKA and p-CREB in cerebrum nuclear fraction of hens at 8 h and on 10 days after administration (Damodaran *et al.*, 2009). The finding indicated that PKA and p-CREB might play an important role in the initiation, progression, and development of OPIDN, while the inhibition and subsequent aging of NTE has been proposed to be an initiating event in OPIDN (Johnson, 1974, 1977; Hou *et al.*, 2008). These studies indicated that inhibition of PKA and p-CREB causing OPIDN might be associated with NTE inhibition; however, whether cAMP/PKA/CREB might be involved in OPIDN via inhibition of NTE needs to be further studied.

In conclusion, we provided direct evidence for the first time that CREB can bind to the promoter region of *NTE* gene and upregulate NTE expression in HeLa cells. We also showed that cAMP/PKA signaling is involved in the upregulation of NTE expression by CREB.

Acknowledgments

This work was supported in part by the grants from the National Natural Science Foundation of China (No. 30870537, 31071919) and the National Basic Research Program of China (No. 2012CB114100). We thank Ms. H. Huang for polishing the English of the article.

Disclosure Statement

No competing financial interests exist.

References

- Akassoglou, K., Malester, B., Xu, J., Tessarollo, L., Rosenbluth, J., and Chao, M.V. (2004). Brain-specific deletion of neuropathy target esterase/Swiss cheese results in neurodegeneration. *Proc Natl Acad Sci U S A* **101**, 5075–5080.
- Chang, P.A., and Wu, Y.J. (2009). Motor neuron diseases and neurotoxic substances: a possible link? *Chem Biol Interact* **180**, 127–130.
- Chang, P.A., and Wu, Y.J. (2010). Neuropathy target esterase: an essential enzyme for neural development and axonal maintenance. *Int J Biochem Cell Biol* **42**, 573–575.
- Chen, J.X., Long, D.X., Hou, W.Y., Li, W., and Wu, Y.J. (2010). Regulation of neuropathy target esterase by the cAMP/protein kinase A signal. *Pharmacol Res* **62**, 259–264.
- Chen, J.X., Xu, L.L., Mei, J.H., Yu, X.B., Kuang, H.B., Liu, H.Y., Wu, Y.J., and Wang, J.L. (2012). Involvement of neuropathy

- target esterase in tri-ortho-cresyl phosphate-induced testicular spermatogenesis failure and growth inhibition of spermatogonial stem cells in mice. *Toxicol Lett* **211**, 54–61.
- Chen, R., Chang, P.A., Long, D.X., Liu, C.Y., Yang, L., and Wu, Y.J. (2007). G protein beta2 subunit interacts directly with neuropathy target esterase and regulates its activity. *Int J Biochem Cell Biol* **39**, 124–132.
- Conkright, M.D., Guzmán, E., Flechner, L., Su, A.I., Hogenesch, J.B., and Montminy, M. (2003). Genome-wide analysis of CREB target genes reveals a core promoter requirement for cAMP responsiveness. *Mol Cell* **11**, 1101–1108.
- Dammer, E.B., Leon, A., and Sewer, M.B. (2007). Coregulator exchange and sphingosine-sensitive cooperativity of steroidogenic factor-1, general control nonderepressed 5, p54, and p160 coactivators regulate cyclic adenosine 3',5'-monophosphate-dependent cytochrome P450c17 transcription rate. *Mol Endocrinol* **21**, 415–438.
- Damodaran, T.V., Gupta, R.P., Attia, M.K., and Abou-Donia, M.B. (2009). DFP initiated early alterations of PKA/p-CREB pathway and differential persistence of beta-tubulin subtypes in the CNS of hens contributes to OPIDN. *Toxicol Appl Pharmacol* **240**, 132–142.
- Gonzalez, G.A., and Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675–680.
- Hou, W.Y., Long, D.X., Wang, H.P., Wang, Q., and Wu, Y.J. (2008). The homeostasis of phosphatidylcholine and lysophosphatidylcholine was not disrupted during tri-o-cresyl phosphate-induced delayed neurotoxicity in hens. *Toxicology* **252**, 56–63.
- Impey, S., McCorkle, S.R., Cha-Molstad, H., Dwyer, J.M., Yochum, G.S., Boss, J.M., McWeeney, S., Dunn, J.J., Mandel, G., and Goodman, R.H. (2004). Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. *Cell* **119**, 1041–1054.
- Johnson, M.K. (1974). The primary biochemical lesion leading to the delayed neurotoxic effects of some organophosphorus esters. *J Neurochem* **23**, 785–789.
- Johnson, M.K. (1977). Improved assay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. *Arch Toxicol* **37**, 113–115.
- Li, Y., Dinsdale, D., and Glynn, P. (2003). Protein domains, catalytic activity, and subcellular distribution of neuropathy target esterase in Mammalian cells. *J Biol Chem* **278**, 8820–8825.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.
- Lush, M.J., Li, Y., Read, D.J., Willis, A.C., and Glynn, P. (1998). Neuropathy target esterase and a homologous *Drosophila* neurodegeneration-associated mutant protein contain a novel domain conserved from bacteria to man. *Biochem J* **332**, 1–4.
- Mantamadiotis, T., Papalexis, N., and Dworkin, S. (2012). CREB signalling in neural stem/progenitor cells: Recent developments and the implications for brain tumour biology. *Bioessays* **34**, 293–300.
- Mayr, B., and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* **2**, 599–609.
- Moser, M., Li, Y., Vaupel, K., Kretzschmar, D., Kluge, R., Glynn, P., and Buettner, R. (2004). Placental failure and impaired vasculogenesis result in embryonic lethality for neuropathy target esterase-deficient mice. *Mol Cell Biol* **24**, 1667–1679.
- Muhlig-Versen, M., da Cruz, A.B., Tschape, J.A., Moser, M., Buttner, R., Athenstaedt, K., Glynn, P., and Kretzschmar, D. (2005). Loss of Swiss cheese/neuropathy target esterase activity causes disruption of phosphatidylcholine homeostasis and neuronal and glial death in adult *Drosophila*. *J Neurosci* **25**, 2865–2873.
- Read, D.J., Li, Y., Chao, M.V., Cavanagh, J.B., and Glynn, P. (2009). Neuropathy target esterase is required for adult vertebrate axon maintenance. *J Neurosci* **29**, 11594–11600.
- Rexach, J.E., Clark, P.M., Mason, D.E., Neve, R.L., Peters, E.C., and Hsieh-Wilson, L.C. (2012). Dynamic O-GlcNAc modification regulates CREB-mediated gene expression and memory formation. *Nat Chem Biol* **8**, 253–261.
- Rudolph, D., Tafuri, A., Gass, P., Hämmerling, G.J., Arnold, B., and Schütz, G. (1998). Impaired fetal T cell development and perinatal lethality in mice lacking the cAMP response element binding protein. *Proc Natl Acad Sci U S A* **95**, 4481–4486.
- Sands, W.A., and Palmer, T.M. (2008). Regulating gene transcription in response to cyclicAMP elevation. *Cell Signal* **20**, 460–466.
- Williams, D.G. (1983). Intramolecular group transfer is a characteristic of neurotoxic esterase and is independent of the tissue source of the enzyme. *Biochem J* **209**, 817–829.
- Winrow, C.J., Hemming, M.L., Allen, D.M., Quistad, G.B., Casida, J.E., and Barlow, C. (2003). Loss of neuropathy target esterase in mice links organophosphate exposure to hyperactivity. *Nat Genet* **33**, 477–485.
- Wu, H., Li, T., Zeng, M., and Peng, T. (2012). Herpes simplex virus type 1 infection activates the Epstein-Barr virus replicative cycle via a CREB-dependent mechanism. *Cell Microbiol* **14**, 546–559.
- Yamamizu, K., Matsunaga, T., Katayama, S., Kataoka, H., Takayama, N., Eto, K., Nishikawa, S., and Yamashita, J.K. (2012). PKA/CREB signaling triggers Initiation of endothelial and hematopoietic cell differentiation via Etv2 induction. *Stem Cells* **30**, 687–696.
- Zaccheo, O., Dinsdale, D., Meacock, P.A., and Glynn, P. (2004). Neuropathy target esterase and its yeast homologue degrade phosphatidylcholine to glycerophosphocholine in living cells. *J Biol Chem* **279**, 24024–24033.
- Zhang, X., Odom, D.T., Koo, S.H., Conkright, M.D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E., Kadam, S., Ecker, J.R., Emerson, B., Hogenesch, J.B., Unterman, T., Young, R.A., and Montminy, M. (2005). Genome-wide analysis of cAMP-responsive element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proc Natl Acad Sci U S A* **102**, 4459–4464.

Address correspondence to:

Yi-Jun Wu, PhD

Laboratory of Molecular Toxicology

State Key Laboratory of Integrated Management

of Pest Insects and Rodents

Institute of Zoology

Chinese Academy of Sciences

1-5 Beichenxi Road

Chaoyang District

Beijing 100101

People's Republic of China

E-mail: wuyj@ioz.ac.cn

Received for publication August 21, 2012; received in revised form January 24, 2013; accepted February 18, 2013.