RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2006.32.115

ScienceAsia 32 (2006): **115-119**

Impairment of Neurotrophin-3-Induced Phosphorylation of cAMP Response Element-Binding Protein Caused by Interleukin-1β in Cultured Cortical Neurons

Rungtip Soi-ampornkul[a], Liqi Tong[b], Wipawan Thangnipon[a*], Carl W. Cotman[b] and Robert Balazs[b]

[a] Neuro-Behavioral Biology Center, Institute of Science and Technology for Research and Development, Mahidol University, Salaya Campus, Nakhon Pathom, 73170, Thailand.
[b] University of California Irvine, Institute for Brain Aging and Dementia, Irvine, California, 92697-4540, U.S.A.

* Corresponding author, E-mail: grwtn@mahidol.ac.th

Received 19 Nov 2004
Accepted 23 Nov 2005

ABSTRACT: Increased expression of interleukin-1β (IL-1β) has been implicated as a driving force in neurodegenerative cascades that underly the formation of neuritic plaques and neurofibrillary tangles, and the accompanying neuronal cell injury and loss. In view of the critical role played by cAMP-response element-binding protein (CREB) phosphorylation in neuronal plasticity, IL-1β might contribute to the decline in cognitive functions preceding the overt manifestation of pathologies in the neurodegenerative diseases such as Alzheimer's disease (AD). In this study we have explored the effects of IL-1β on neurotrophin-3 (NT-3) mediated signal transduction in neuronal cell cultures of the rat cerebral cortex. Pretreatments with 10 and 50 ng/ml of IL-1β significantly suppressed the effects of NT-3 by decreasing the level of phosphorylated CREB (P-CREB) to 89% and 71% ($p< 0.05$) of the level with NT-3 alone, respectively. This suggests that IL-1β may interfere with the functions of NT-3 in the early course of neuronal degeneration. We found that 5 μg/ml of interleukin-1 receptor antagonist (IL-1ra) blocked IL-1β, with a significant increase in CREB phosphorylation when IL-1ra was added along with IL-1β. Our study also indicates that NT-3 is effective in increasing the levels of P-CREB, which may play a crucial role in signal transduction processes that mediate neuronal plasticity.

KEYWORDS: Signal transduction, Neurotrophin-3, Interleukin-1β, Alzheimer's disease.

INTRODUCTION

The neuropathological characteristics of Alzheimer's disease (AD) include the extracellular deposition of amyloid β-peptide (Aβ) associated with a robust inflammatory response that might contribute to neuronal degeneration[1]. The view that inflammatory processes play an important role in the pathogenesis of AD has been supported by epidemiological studies showing that anti-inflammatory drugs result in a slower progression of the disease[2], and that a non-steroidal anti-inflammatory drug, ibuprofen, suppresses amyloid plaque deposition and interleukin-1 (IL-1) expression in a transgenic model of AD[3]. Because of the protractive and progressive nature of the disease, relatively mild inflammatory processes, including somewhat elevated levels of proinflammatory cytokines such as IL-1, might be present in the brain for extended periods before the florid manifestation of the disease.

IL-1 is a pluripotent proinflammatory cytokine that is a potent activator of host defense responses to infection and injury both in the periphery and in the CNS[4]. In particular, IL-1 exacerbates damage in the CNS resulting from acute insults, such as cerebral ischemia and trauma. Circumstantial evidence is consistent with a similar role in chronic neurological diseases, such as multiple sclerosis, Parkinson's disease and AD[5]. Furthermore, recent genetic studies have highlighted the relevance of IL-1 in AD pathogenesis, showing that specific polymorphisms in the IL-1 gene cluster are associated with a greatly increased risk for AD, especially for the earlier onset form of the disease[6]. The IL-1 family comprises agonists, such as IL-1α and IL-1β, which are the products of separate genes believed to exert identical actions via binding to the IL-1RI receptor that requires association with an accessory protein, AcP, for signal transduction. The third member of the family is a competitive interleukin-1 receptor antagonist (IL-1ra) that selectively blocks the action of the IL-1 agonists. There is general consensus that after the insults, IL-1 has adverse effects on the brain *in vivo* but observations on cultured neurons are less clear. It

seems that IL-1 is neurotoxic only at high concentrations and after relatively long exposure,[7] and in fact it has been reported that at lower concentrations the cytokine provides protection against excitotoxic neuronal death. It would appear that the protective effect is mediated by trophic factors released from glial cells present in the mixed neural cultures upon IL-1 exposure[8]. Neurotrophins mediate the survival and differentiation of specific neuronal populations and exert important influences on synaptic transmission and plasticity primarily through their activation of tyrosine kinase (Trk) receptors[9,10]. In the cerebral cortex, neurons are particularly responsive to brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), the effects of which are mediated via TrkB and TrkC receptors, respectively. Ligand binding to Trk receptors triggers signal transduction through the major cellular signaling pathways, including the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, which has been implicated in diverse physiological functions, including promoting synaptic plasticity[11]. Neurotrophin-induced signaling also activates specific gene transcriptions mediated, in part, through the activation of cyclic AMP response element binding protein (CREB)[12]. CREB-induced gene transcription plays a critical role in learning and memory processes in different species, from *Drosophila* to mammals[13,14] and has survival-promoting features[15,16].

In the present study, we tested the hypothesis that IL-1β under conditions at which it does not compromise the viability of neurons interferes with critical signal transduction processes that mediate plastic neuronal changes, by examining the effect of IL-1β on the NT-3-induced activation of CREB in cultured cerebral cortical neurons.

Materials and Methods

Materials

All cell culture media and supplements were purchased from Life Technologies, Inc. (Gaithersburg, MD, U.S.A.). Human recombinant NT-3, human recombinant IL-1β, and IL-1ra were purchased from Pepro Tech (Rocky Hill, NJ, U.S.A.). The BCA protein assays kits were obtained from Pierce (Rochford, IL, U.S.A.) and enhance chemiluminescence (ECL) reagent was obtained from Amersham Pharmacia (Piscaway, NJ, U.S.A.). The following antibodies were used for the biochemical studies: phosphorylated CREB (P-CREB) (detects CREB phosphorylated at Ser133, 1:2,000), and total CREB (T-CREB, which recognizes both phospho- and dephospho-related CREB, 1:2,000), from Upstate (Charlottesville, VA, U.S.A.). Goat anti-rabbit IgG conjugated with horseradish peroxidase was purchased from Vector Laboratories (Burlingame, CA, U.S.A.). Electrophoresis chemicals were from Bio-RAD (Hercules, CA, U.S.A.).

Cell Culture

Primary cultures of dissociated cerebral cortical neurons were prepared from the brain of embryonic day-18 (E 18) rat fetuses as described previously[17]. Cells plated at 2.5×10^4 cells/cm^2 were cultured in poly-L-lysine treated multiwell plates and maintained in serum-free optimal Dulbecco's modified Eagles medium (DMEM) supplemented with B-27, which contained several antioxidants, such as vitamin B, vitamin C, catalase, glutathione and superoxide dismutase (Invitrogen, Carlsbad, CA, U.S.A.). Cultures were maintained for 5 days in vitro (5 DIV) before treatments. Cultures were incubated in DMEM in the presence or absence of human recombinant NT-3 at the concentrations and for the lengths of time indicated. Human recombinant IL-1β, was dissolved in DMEM and used after one freeze-thaw cycle. Cells were preincubated for 2 h with IL-1β or IL-1ra before the addition of NT-3. After 15 min of incubation with NT-3, the cells were lysed and subjected to electrophoresis.

Gel Electrophoresis and Western Blotting

Cultures of cortical neurons were lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 20 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulphonyl fluoride, 0.7 μg/ml pepstatin and 1 μg/ml leupeptin. The lysates were centrifuged at 12,000 g at 4°C for 30 min, and then the protein concentrations of the clarified lysate were determined by the BCA protein assay (Pierce, Rockford, IL, U.S.A.). An aliquot of the lysates containing 10 μg of protein was diluted in sample buffer [0.125 mM Tris-HCl, pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS, and 10% (v/v) β-mercaptoethanol]. After boiling 5 min the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels. Proteins were then electro-transferred to nitrocellulose membranes. Membranes were incubated at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% nonfat milk for 60 min to block nonspecific binding. Following incubation with primary antibodies specific for either phosphorylated CREB (P-CREB) or total CREB at 1:2000 dilution, the membranes were washed in TBS with Tween 20 and then incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG at 1:2000 dilution in the blocking solution) for 60 min. Blots were then washed four-times with TBS with Tween 20. Immunolabeling was detected by ECL reagent (Amersham Phamarcia) according to the manufactures recommended conditions. Immunoreactivity was

quantified using a densitometric analysis program, NIH image (www.scioncorp.com).

Statistical Analysis

Three independent experiments were conducted in all studies and all assay conditions were performed in triplicate. Data were analyzed by one-way ANOVA using Fisher-PSD for *post hoc* comparisons. Differences were considered statistically significant with p values less than 0.05.

Results

Effect of IL–1β on NT-3-induced activation of P-CREB

Before exploring the effect of IL-1β on NT-3-induced signaling, the responses of cortical neurons to this neurotrophin were examined. The effect of IL-1β on NT-3-induced CREB activation is shown in Fig. 1A and 1B. Preincubation with the 10 and 50 ng/ml IL-1β, which are in the subtoxic range, for 2 h resulted in a concentration-dependent decrease in the effect of NT-3 on P-CREB levels without affecting the total amount of CREB (T-CREB), with decreases in the level of P-CREB to 89% and 71% of that in the NT-3 treated control, respectively.

Interference of NT-3-induced P-CREB activation by IL-1ra

The effects of IL-1ra on the IL-1β inhibition of the NT-3-induced increase in P-CREB were then examined. Cultures at 5 DIV were preincubated with 50 ng/ml of IL-1β for 2 h and then incubated in the presence or absence of 50 ng/ml of NT-3 for 15 min. Pretreatment

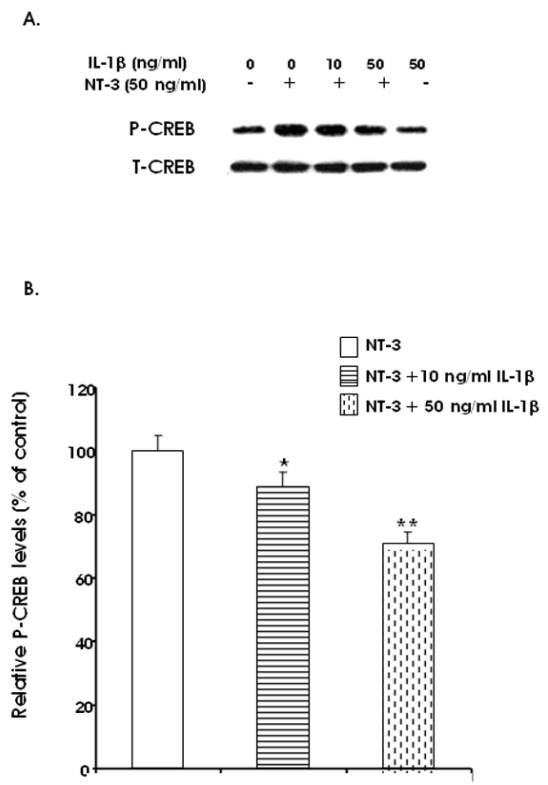

Fig 1. Pretreatment with 10 or 50 ng/ml IL–1β suppressed the NT-3-induced increase in P-CREB in cultured cortical neurons. Western blots showing the analysis of phosphorylated CREB (P-CREB) and total CREB (T-CREB) are displayed in (A). Each lane corresponds to 10 μg of cell lysate protein. Quantification of pretreatment effects with 0, 10 or 50 ng/ml IL–1β is shown in (B). Estimations are expressed in terms of P-CREB levels obtained in NT-3-exposed cultures. Bars represent mean ± SEM (n=3). Significant decreases when compared with NT-3 treated cultures are indicated by $*p < 0.05$ and $**p < 0.01$, (ANOVA, Fisher's PLSD as the *post hoc* test).

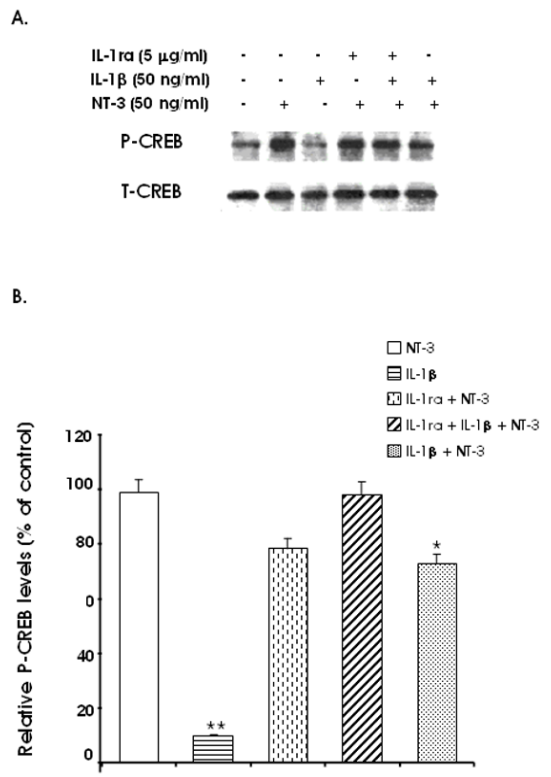

Fig 2. Interference of NT-3-induced P-CREB activation by IL-1ra. Pretreatment with 5 μg/ml IL-1ra suppressed the effects of IL-1β by increasing P-CREB. Western blots showing analysis of phosphorylated CREB (P-CREB) and total CREB (T-CREB) are displayed in (A). Each lane corresponds to 10 μg of cell lysate protein. Quantification of pretreatment effects with 10 or 50 ng/ml IL–1β is shown in (B). Estimations are expressed in terms of P-CREB levels obtained in the NT-3-exposed cultures. Bars represent mean ± SEM (n=3). Significant decreases when compared with NT-3 treated cultures are indicated by $*p < 0.05$ and $**p < 0.01$, (ANOVA, Fisher's PLSD as the *post hoc* test).

with IL-1β significantly suppressed the NT-3 induced increase in P-CREB, with a decrease of 30% in P-CREB compared with that with NT-3 alone (Fig. 2A and 2B). It was also found that 5 μg/ml IL-1ra blocked IL-1β by significantly increasing the level of P-CREB, which were 99% of the NT-3 treated control when IL-1ra was added before adding IL-1β. These data indicated that IL-1ra blocked the effects of IL-1β on NT-3-induced P-CREB.

Discussion

CREB is one of the targets in transcription factor activation; it has been implicated in processes of neuronal plasticity, including learning and memory. CREB is a key regulator in the induction of gene expression by BDNF, suggesting that CREB plays an important role in mediating neurotrophin responses in neurons[18]. In this study we found that treatment of cortical neurons with NT-3 resulted in a rapid and dramatic stimulation of CREB phosphorylation. IL-1β significantly suppressed this effect of NT-3 with a significant decrease in the level of P-CREB. Pretreatment with 100 μg/ml IL-1ra (1 h before adding IL-1β) was required for optimal inhibition of neuroprotection by IL-1α or IL-1β[19]. Similarly, in this study we added 5 μg/ml of IL-1ra (30 min before adding 50 ng/ml IL-1β) and found that IL-1ra blocked the effect of IL-1β on NT-3, with a significantly increased level of P-CREB. In our experimental conditions, exposure to IL-1ra and NT-3 resulted in reduced level of P-CREB but the reduction was not significant. At present, the mechanism of IL-1β neurotoxicity is unknown, but it appears to be mediated by the IL-1 receptor type I, since application of IL-1ra reduced its neurotoxicity. Similarly, the previous studies have reported that exposure of cultures to 50 ng/ml IL-1ra and 500 ng/ml IL-1β for 24 h before or after exposure to submaximal concentrations of excitatory amino acid significantly improved the neuroprotective effects of IL-1β excitatory amino-acid induced neurodegeneration.

The distribution of NT-3 in the cerebral cortex *in situ* is similar to that of BDNF[20]. Furthermore, in various systems, NT-3 activates similar transduction pathways as BDNF via an NT-3-specific high affinity TrkC receptor[21]. There is general agreement with the evidence that similar signal transduction pathways are activated by BDNF via TrkB as by NT-3 via TrkC[22]. However, compared with BDNF, the suppression of the neuronal activity-induced P-CREB levels by $A\beta_{1-42}$ also results in a decrease in the transcription of CREB target genes, as demonstrated by the reduction of BDNF expression[23]. BDNF has been shown to participate in certain forms of long-term potentiation (LTP) in learning and memory[24]. Compared with BDNF, the information about NT-3-induced signaling in cerebral cortical neurons is limited. Certain aspects of NT-3-induced signal transduction have been examined and it was found that the activation of MAPK/ERK, phosphatidylinositol 3-kinase (PI3-K) and the CREB transcription factor were similar to those observed in BDNF. Although IL-1β does not directly compromise or promote the survival of neurons under resting conditions, and does not induce the activation of MAPK/ERK, the cytokine has an adverse influence on cells that are stimulated[25] by exposure to NT-3, as shown in the present work. On the other hand, IL-1β interferes with the activation of signal transduction induced by NT-3, in that IL-1β suppresses the phosphorylation of CREB. The effect of the cytokine is specific, as IL-1ra blocked the suppression of NT-3-induced phosphorylation of CREB. It would be expected that the protracted exposure of neurons to elevated IL-1β levels, as it seems to occur in AD even before the manifestation of the florid symptoms of the disease, might contribute to a cognitive deficit at an early stage prior to or along with neuronal degeneration.

Acknowledgements

This work was supported by the Thailand Research Fund through Thai Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0191/2542) and the University of California Irvine. Institute for Brain Aging and Dementia, Irvine, California, U.S.A.

References

1. Akiyama H, Barger S, Barnum S and Bonnie B (2000) Inflammation and Alzheimer's disease. *Neurobiol Aging* **21**, 383-421.
2. Breitner JC, Gau BA, Welsh KA, Plassman BL, McDonald WM, Helms MJ and Anthony JC (1994) Inverse association of anti-inflammatory treatments and Alzheimer's disease: initial results of a co-twin control study. *Neurology* **44**, 227-32.
3. Lim GP, Yang F, Chu T, Gahtan E, Ubeda O and Beech W (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. *J Neurosci* **20**, 5709-14.
4. Rothwell NJ and Luheshi GN (2000) Interleukin 1 in the brain: biology, pathology and therapeutic target. *Trends Neurosci* **23**, 618-25.
5. Mrak RE and Griffin WS (2001) Interleukin-1, neuroinflammation, and Alzheimer's disease. *Neurobiol Aging* **22**, 903-8.
6. Grimaldi LM, Casadei VM, Ferri C, Veglia F, Licastro F and Annoni G (2000) Association of early-onset Alzheimer's disease with an interleukin-1 alpha gene polymorphism. *Ann Neurol* **47**, 361-5.
7. Araujo DM and Cotman CW (1995) Differential effects of interleukin-1 beta and interleukin-2 on glia and hippocampal neurons in culture. *Int J Dev Neurosci* **13**, 201-12.
8. Strijbos PJ and Rothwell NJ (1995) Interleukin-1 beta attenuates excitatory amino acid-induced neurodegeneration in vitro: involvement of nerve growth factor. *J Neurosci* **15**,

3468-74.
9. Kaplan DR and Miller FD (2000) Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol* **10**, 381-91.
10. Thoenen H (2000) Neurotrophins and activity-dependent plasticity. *Prog Brain Res* **128**, 183-91.
11. Sweatt JD (2001) The neuronal MAP kinase cascades: a biochemical signal integration system subserving synaptic plasticity and memory. *J Neurochem* **76**, 1-10.
12. Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM and Greenberg ME (1997) CREB: a major mediator of neuronal neurotrophin responses. *Neuron* **19**, 1031-47.
13. Yin JC, Wallach JS, Del Vecchio M, Wilder EL, Zhou H and Quinn WG (1994) Induction of a dominant negative CREB transgene specifically blocks long-term memory in *Drosophila*. *Cell* **79**, 49-58.
14. Tully T (1979) Regulation of gene expression and its role in long-term memory and synaptic plasticity. *Proc Natl Acad Sci USA* **94**, 4239-41.
15. Bonni A, Brunet A, West AE, Datta SR, Takasu MA and Greenberg MA (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and independent mechanisms. *Science* **286**, 1358-62.
16. Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y and Sue L (1999) Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am J Pathol* **155**, 853-62.
17. Pike CJ, Burdick D, Walencewicz AJ, Glabe CG and Cotman CW (1993) Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. *J Neurosci* **13**, 1676-87.
18. Numakawa T, Yamagishi S, Adachi N, Matsumoto T, Yokomaku D and Yamada M (2002) Brain-derived neurotrophic factor-induced potentiation of Ca^{2+} oscillations in developing cortical neurons. *J Biol Chem* **277**, 6520-9.
19. Carlson NG, Wieggel WA, Chen J, Bacchi A, Rogers SW and Gahring LC (1999) Inflammatory cytokines IL-1 alpha, IL-1 beta, IL-6 and TNF-alpha impart neuroprotection to an excitotoxin through distinct pathways. *J Immunology* **163**, 3963-8.
20. Pitts AF and Miller MW (2000) Expression of nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 in the somatosensory cortex of the mature rat: coexpression with high-affinity neurotrophin receptors. *J Comp Neurol* **418**, 241-54.
21. Friedman WJ and Greene LA (1999) Neurotrophin signaling via Trks and p75. *Exp Cell Res* **253**, 131-42.
22. Friedman HV, Bresler T, Garner CC and Ziv NE (2000) Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron* **27**, 57-69.
23. Tong L, Balazs R, Thornton PL and Cotman CW (2004) *b*-Amyloid peptide at sublethal concentrations downregulates brain-derived neurotrophic factor function in culture cortical neurons. *J Neurosci* **24**, 6799-809.
24. Huang EJ and Reichardt LF (2001) Neurotrophins: role in neuronal development and function. *Ann Rev Neurosci* **24**, 677-736.
25. Srinivasan D, Yen JH, Joseph DJ and Friedman W (2004) Cell type-specific interleukin-1 beta signaling in the CNS. *J Neurosci* **29**, 6482-8.