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cAMP/PKA enhances interleukin-1β-induced interleukin-6 synthesis through STAT3 in glial cells

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ABSTRACT

We previously reported that interleukin (IL)-1β induces IL-6 synthesis via activation of the IκB/NFκB pathway, p38 mitogen-activated protein (MAP) kinase, stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK), and signal transducer and activator of transcription (STAT)3, but not p44/p42 MAP kinase in rat glioma cell line, C6 cells and that cAMP enhances the IL-6 synthesis. However, the details behind enhancement of IL-1β-induced IL-6 synthesis by cAMP remain to be elucidated. In the present study, we investigated the exact mechanism of cAMP underlying the amplification of IL-1β-induced IL-6 synthesis in C6 cells. 8-Bromo cAMP significantly enhanced IL-1β-induced STAT3 phosphorylation without affecting phosphorylation of IκB, p38 MAP kinase or SAPK/JNK. In addition, we found that forskolin, a direct activator of adenylyl cyclase, significantly enhanced IL-1β-induced STAT3 phosphorylation. Janus family of tyrosine kinase (JAK) inhibitor I markedly suppressed the amplification by 8-bromo cAMP of IL-1β-induced IL-6 release. IL-1β induced JAK2 phosphorylation, and FLLL32, a specific JAK2 inhibitor, significantly reduced IL-1β-stimulated IL-6 release. 4-Cyano-3-methylisoquinoline, an inhibitor of protein kinase A (PKA), significantly attenuated the enhancing effect of 8-bromo cAMP on IL-1β-induced STAT3 phosphorylation. 8-Bromo cAMP markedly induced JAK2 phosphorylation. PKA siRNA transfection reduced enhancement of IL-1β-induced IL-6 release by 8-bromo cAMP. In conclusion, our results strongly suggest that the adenylyl cyclase/cAMP/PKA pathway upregulates IL-1β-induced IL-6 synthesis through enhancement of the JAK2/STAT3 pathway in C6 glioma cells.

1. Introduction

Adenylyl cyclase converts ATP to cAMP, resulting in activation of protein kinase A (PKA) [1–3]. In the brain, the adenylyl cyclase/cAMP pathway contributes to a variety of neural functions including synaptic plasticity, long-term potentiation, learning, memory, sensitization of nociceptors and neurodegeneration [1–3]. For example, the decrease in Ca^{2+}-stimulated cAMP production correlates with a decrease in long-term potentiation and deficiency in spatial memory, and overexpression of adenylyl cyclase in the brain enhances recognition memory and long-term potentiation [3]. Elevation of cAMP level leads to hyperalgesia [4] and lack of cAMP activity decreases pain response [3].

Inflammation is generally an advantage response of an organism to infection, however, when prolonged or inappropriate, it can be tending to cause harm. Brain inflammation is implicated in a number of acute or chronic central nervous system (CNS) diseases such as stroke, traumatic brain injury, multiple sclerosis and Alzheimer's disease [5–9]. Cytokines act as central mediators of the inflammatory response. Among them, interleukin (IL)-1 plays a pivotal role. In healthy brain, the IL-1 family is expressed at low levels and regulates important physiological functions such as sleep, memory and long-term potentiation [5,6]. On the other hand, IL-1 expression increases in acute CNS injury and initiates inflammatory response through action on non-neuronal cells [6,10]. In astrocytes, pathological increased IL-1 activates the IκB/NFκB pathway and the mitogen-activated protein (MAP) kinase superfamily, such as p38 MAP kinase, p44/p42 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) [6,10]. One of the responses of astrocytes for IL-1 is the synthesis and release of a variety of secondary inflammatory mediators, such as cytokines (IL-6, IL-8 and tumor necrosis factor-α), chemokines, prostaglandin (PG)s and neurotoxic factors [6–7,10]. However, the exact mechanism on how these mediators are synthesized has not yet been precisely clarified.

Abbreviations: 8-bromo cAMP, adenosine-3′,5′-cyclic monophosphate, 8-bromo-, sodium salt; CMI, 4-cyano-3-methylisoquinoline; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; EPAC, exchange proteins directly activated by cAMP; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; JAK, Janus family of tyrosine kinase; MAP, mitogen-activated protein; PGE, prostaglandin; PKA, protein kinase A; SAPK/JNK, stress-activated protein kinase/c-*Jun* N-terminal kinase; SDS, sodium dodecyl sulfate; STAT, signal transducer and activator of transcription.

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We have previously reported that IL-1β induces IL-6 synthesis via activation of the IκB/NFκB pathway, p38 MAP kinase, SAPK/JNK and signal transducer and activator of transcription (STAT)3, but not p44/p42 MAP kinase in rat glioma cell line, C6 cells [11]. We additionally demonstrated that cAMP amplifies IL-1β-induced IL-6 mRNA expression and IL-6 release in C6 cells [12]. It has been shown that forskolin, an activator of adenylyl cyclase, enhances IL-1-induced IL-6 synthesis in thyroid cells and AP-1 and DRE binding sites are involved in this effect [13]. Also in neuronal cells, cAMP has been reportedly to upregulate IL-1β-induced IL-6 release [14]. However, the molecular mechanism underlying enhancement of IL-1-induced IL-6 synthesis by cAMP is not elucidated. In the present study, we investigated how the adenylyl cyclase/cAMP pathway enhances IL-1-induced IL-6 synthesis in C6 glioma cells.

2. Material and methods

2.1. Materials

IL-6 enzyme-linked immunosorbent assay (ELISA) kit and IL-1β were obtained from R&D System (Minneapolis, MN). Prostaglandin (PG)E_2 and forskolin were obtained from Sigma Chemical Co. (St. Louis, MO). Adenosine-3′,5′-cyclic monophosphate, 8-bromo-, sodium salt (8-bromo cAMP), Janus family of tyrosine kinase (JAK) inhibitor I, 4-cyano-3-methylisoquinoline (CMI) and FLLL32 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific IκB, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK, SAPK/JNK, phospho-specific STAT3, STAT3, phospho-specific JAK2 and STAT4 antibodies were purchased from Cell Signaling (Beverly, MA). Phospho-specific JAK1 antibodies were purchased from Millipore Corporation (Temecula, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An enhanced chemiluminescence Western blotting detection system was obtained from GE Healthcare UK, Ltd. (Buckinghamshire, England). Other materials and chemicals were obtained from commercial sources. JAK inhibitor I, forskolin and FLLL32 were dissolved in dimethyl sulfoxide. PGE_2 was dissolved in ethanol. The maximum concentration of dimethyl sulfoxide or ethanol was 0.1%, which did not affect the assay for IL-6 or the detection of protein level using Western blot analysis.

2.2. Cell culture

Rat C6 glioma cells, obtained from the American Type Culture Collection (Rockville, MD), were seeded into 35-mm (5×10^4 cells/dish) or 90-mm (2×10^5 cells/dish) diameter dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2/95% air. After 6 days, the medium was exchanged for serum-free DMEM. The cells were then used for experiments after 24 h. The cells were pretreated with 8-bromo cAMP, forskolin or FLLL32 for 60 min before IL-1β stimulation. When indicated, the cells were treated with JAK inhibitor I or CMI for 60 min before 8-bromo cAMP pretreatment.

2.3. Assay for IL-6

Cultured cells (35-mm diameter dishes) were stimulated with 10 ng/ml IL-1β in serum-free DMEM for 36 h. The conditioned medium was collected at the end of the incubation, and IL-6 concentration was measured using an ELISA kit. The absorbance of each sample at 450 nm and 540 nm was measured with a Multiscan JX ELISA reader (Thermo Labsystems, Helsinki, Finland). Absorbance was corrected with reference to a standard curve.

2.4. Western blot analysis

Cultured cells (90-mm diameter dishes) were stimulated with 10 ng/ml IL-1β in serum-free DMEM for the indicated periods. The cells were washed twice with phosphate-buffered saline, and then lysed and sonicated in a lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The samples were separated by SDS-polyacrylamide gel electrophoresis by the method of Laemmli [15] in 7.5 or 10% polyacrylamide gels. Western blot analysis was performed using phospho-specific IκB antibodies, GAPDH, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific STAT3 antibodies, STAT3 antibodies, phospho-specific JAK1 antibodies, phospho-specific JAK2 antibodies or STAT4 antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as secondary antibodies. Peroxidase activity on polyvinylidene difluoride membrane was visualized on X-ray film by utilizing an enhanced chemiluminescence Western blotting detection system.

2.5. Densitometric analysis

The densitometric analysis was performed using scanner and image analysis software (ImageJ ver.1.32). The background-subtracted signal intensity of each phosphorylation signal was normalized to the respective total protein or GAPDH, and plotted as the fold increase in comparison to control cells without stimulation.

2.6. siRNA transfection

Cells were transfected with negative control siRNA (Silencer Negative Control no. 1; Ambion), PKA siRNA (s130330), EPACs siRNA (s133229) or JAK2 siRNA (s127977), (Silencer Predesigned siRNA, Thermo Fisher) utilizing siLentFect (Bio-Rad) according to the manufacturer's protocol. In brief, cells (5×10^4 cells/dish) were seeded into 35-mm diameter dishes in DMEM containing 10% fetal bovine serum. The cells were then incubated at 37 °C with 50 nM or 70 nM siRNA-siLentFect complexes. After 72 h, the medium was exchanged to serum free DMEM. The cells were then used after 24 h.

2.7. Statistical analysis

The data were analyzed by ANOVA followed by Bonferroni's method for multiple comparisons between pairs. Values of $P < 0.05$ were considered to statistically significant. All data are presented as the mean ± SD of triplicate determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effect of PGE_2 on IL-1β-induced IL-6 release from C6 cells

It has been reported that cAMP amplifies IL-1β-induced IL-6 release from C6 cells [12,16]. In our previous study [12], we have shown that 8-bromo cAMP enhances IL-1β-induced IL-6 mRNA expression in C6 cells. In order to investigate whether an endogenous receptor ligand that elevates cAMP has a similar effect to cAMP, we examined effect of PGE_2 on IL-1β-stimulated IL-6 release from C6 cells. PGE_2, a major endogenous eicosanoid in the CNS, is a product by action of cyclooxygenase on arachidonic acid and increases intracellular cAMP formation in astrocytes [17–19]. We found that 1 μM of PGE_2, which by itself did not affect the IL-6 levels, truly enhanced IL-1β-induced IL-6 release from C6 cells. PGE_2 caused an approximate 40% increase in the IL-1β-effect.