

Thromboxane A₂ Promotes Interleukin-6 Biosynthesis Mediated by an Activation of Cyclic AMP-Response Element-Binding Protein in 1321N1 Human Astrocytoma Cells

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ABSTRACT

1321N1 human astrocytoma cells express thromboxane A₂ (TXA₂) receptors (TP). However, physiological consequences of TXA₂ signaling in glial cells remain unclear. Herein, we show that TXA₂ promotes interleukin-6 (IL-6) biosynthesis in glial cells. A TP agonist, 9,11-dideoxy-9 α ,11 α -methanoepoxy-prosta-5Z,13E-dien-1-oic acid (U46619), enhanced IL-6 production in both 1321N1 cells and cultured mouse astrocytes. It has been shown that IL-6 gene expression is regulated by various transcription factors. Among them, we found a significant increase in cyclic AMP-response element-binding protein (CREB) activity with its phosphorylation at Ser133 by U46619 in 1321N1 cells. Although U46619 increased IL-6 promoter activity, a mutation at cyclic AMP-response element (CRE) on the promoter clearly suppressed the effect, suggesting that CRE is involved in U46619-induced IL-6 expression. Furthermore, both CREB and IL-6 promoter activities were suppressed by SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-

pyridyl)-1H-imidazole], a p38 mitogen-activated protein kinase (MAPK) inhibitor, and H89 [N-[2-(4-bromocinnamylamino)-ethyl]-5-isoquinoline], a protein kinase A (PKA) inhibitor, indicating involvements of p38 MAPK and PKA in CREB activation and IL-6 expression. To determine which G-proteins are implicated in the U46619-induced IL-6 synthesis, the interfering mutants of G α_q , G α_{12} , or G α_{13} were overexpressed in 1321N1 cells by adenoviral approach. It is noteworthy that the G α_q or G α_{13} mutant blocked the IL-6 production by U46619. The constitutively active mutant of G α_q , G α_{12} , or G α_{13} enhanced IL-6 production, indicating that G α_q and G α_{13} were involved in U46619-induced IL-6 production. In conclusion, TXA₂ enhances the IL-6 biosynthesis via the PKA p38 MAPK/CREB pathway in 1321N1 cells. IL-6 induction depends on G α_q and G α_{13} as well. This is the first report showing TP-mediated IL-6 production in glial cells.

The central nervous system (CNS) consists mainly of neurons and glial cells. Neurons play a central role in signal transduction by releasing neurotransmitters, whereas glial

cells support the neuronal functions. Glial cells in CNS are generally classified into astrocytes, oligodendrocytes, and microglia. Astrocytes form the blood-brain barrier along with endothelial cells, metabolize neurotransmitters, cause gliosis after brain injury, and regulate extrasynaptic ion and H⁺ concentrations (Somjen, 1988; Ransom et al., 2003). In addition to these roles, they produce neurotrophic factors to promote neuronal survival (prevention of apoptosis), differenti-

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ABBREVIATIONS: CNS, central nervous system; U46619, 9,11-dideoxy-9 α ,11 α -methanoepoxy-prosta-5Z,13E-dien-1-oic acid; CREB, cyclic AMP-response element-binding protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; CRE, cyclic AMP-response element; PKA, protein kinase A; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; GFAP, glial fibrillary acidic protein; DMEM, Dulbecco's modified Eagle's medium; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; Ro20-1724, 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone; KN62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; H89, N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; SQ29548, 1S-[1 α ,2 α (Z),3 α ,4 α]-7-[3-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; PD98059, 2'-amino-3'-methoxyflavone; MSK, mitogen and stress-activated protein kinase; CaMK, calmodulin-dependent kinase; KT5720, (9S,10S,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg':2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester; EGFR, epidermal growth factor receptor; dbcAMP, dibutyl cyclic AMP; ct, carboxyl-terminal; GFP, green fluorescent protein; RSK, ribosomal S6 kinase; AP-1, activator protein-1; NF- κ B, nuclear factor κ B; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PKI, protein kinase inhibitor.

ation, or regeneration. It has been shown that they secrete neurotrophic factors, such as neurotrophin family members (Althaus and Richter-Landsberg, 2000), interleukin-6 (IL-6) (Schwaninger et al., 1997; Van Wagoner and Benveniste, 1999), and glial cell line-derived neurotrophic factor (Lin et al., 1993). We have previously shown that 1321N1 human astrocytoma cells produce nerve growth factor and IL-6 (Obara et al., 2001). These neurotrophic factors have been suggested as potential therapeutic agents for the treatment of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases.

Thromboxane A₂ (TXA₂), a metabolite of arachidonic acid produced by cyclooxygenase and TXA₂ synthetase, is known to induce many cellular responses, including platelet aggregation and contraction in various smooth muscle types (Huang et al., 2004). Because TXA₂ is associated with various diseases, such as thrombogenesis, hypertension, bronchial asthma, and nephrotic syndrome, the antagonist for TXA₂ receptor and inhibitor of its synthesis have been suggested as therapeutic targets (Huang et al., 2004).

We found TXA₂ receptors (TPs) in 1321N1 human astrocytoma cells and rabbit cultured astrocytes (Nakahata et al., 1989, 1992). 1321N1 human astrocytoma cells express both TP isoforms (TP- α and TP- β) (Honma et al., 1998), which are alternatively spliced variants from the same gene. They are seven-transmembrane receptors and have been shown to associate with heterotrimeric GTP-binding proteins, such as G_{q/11}, G_i, G₁₂, G₁₃ and G_h (Offermanns et al., 1994; Nakahata et al., 1995; Allan et al., 1996; Vezza et al., 1999; Gao et al., 2001). In response to TXA₂ analog, an elevation of intracellular Ca²⁺ concentration was observed as a result of phosphatidylinositol-specific phospholipase C activation in 1321N1 cells. TP-mediated extracellular signal-regulated kinase (ERK) activation in differentiated 1321N1 cells depends on the activation of phosphatidylcholine-specific phospholipase C (Kobayashi et al., 2000). It has also been shown that TXA₂ analog elevates cyclic AMP concentration in several cell types, such as Schwann cells (Muja et al., 2001), even though there is currently no evidence for the interaction between TPs and G α_s . In that report, cyclic AMP-response element-binding protein (CREB) is activated, which is assumed to be by a cyclic AMP-dependent mechanism. Thus, the downstream pathway of TPs is complex and has not yet been clarified in detail.

In screening for compounds that enhance neurotrophic factor production in 1321N1 cells, we found that the TXA₂ analog U46619 significantly promoted IL-6 secretion. Herein, we show the mechanism of IL-6 production by TXA₂ in detail.

Materials and Methods

U46619 was obtained from Cayman Chemical (Ann Arbor, MI). Prostaglandin E₂, Ro20-1724, isoproterenol, carbachol, dibutyryl cyclic AMP (dbcAMP), H89, wortmannin, and KN62 were purchased from Sigma-Aldrich (St. Louis, MO). IL-6 enzyme-linked immunosorbent assay (ELISA) kit was from Amersham Biosciences Inc. (Piscataway, NJ). RNA extraction kit was from Nippon Gene Co., Ltd. (Toyama, Japan), and reverse transcription-polymerase chain reaction (RT-PCR) kit was from Toyobo Engineering (Osaka, Japan). Phorbol 12-myristate 13-acetate (PMA), SB203580, and forskolin were from Wako Pure Chemicals (Tokyo, Japan). SQ29548 was from Bristol-Myers Squibb (Tokyo, Japan). KT5720 was from Calbiochem (La Jolla, CA). CRE-luciferase reporter plasmid was from BD Bio-

sciences Clontech (Palo Alto, CA). FuGENE 6 transfection reagent was from Roche Diagnostics (Indianapolis, IN). PD98059, anti-phospho-ERK antibody, anti-phospho-p38 mitogen-activated protein kinase (MAPK) antibody, anti-p38 MAPK antibody, anti-phospho-CREB antibody, anti-CREB antibody, and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody were from Cell Signaling (Beverly, MA). Anti-ERK2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-glial fibrillary acidic protein (GFAP) antibody was from Progen (Heidelberg, Germany). Fluorescein isothiocyanate-conjugated anti-mouse antibody was from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD). Cholera toxin was from List Biological Laboratories Inc. (Campbell, CA).

Cell Culture. 1321N1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (Cell Culture Laboratory, Cleveland, OH), 50 units/ml penicillin, and 50 μ g/ml streptomycin in a 5% CO₂ incubator at 37°C. Mouse astrocytes were prepared from neonatal mouse brains and then cultivated in DMEM supplemented with 10% fetal calf serum, penicillin, and streptomycin (Muller et al., 1984).

IL-6 Sandwich ELISA. IL-6 ELISA was performed as the protocol of IL-6 ELISA kit. In brief, 1321N1 cells were seeded onto six-well plates (1 \times 10⁶ cells/well) and cultivated overnight in the absence of fetal calf serum. The cells were cultured for 24 h in the presence of U46619, and then the conditioned media were collected after centrifugation to remove contaminating cells. These conditioned media were regarded as samples for ELISA and then added to 96-well plates precoated with IL-6 antibody. After incubation, IL-6 antibody conjugated with biotin was added; then, avidin-horseradish peroxidase was added.

Semiquantitative RT-PCR. Total RNA from 1321N1 cells was extracted by using a total RNA extraction kit, and semiquantitative RT-PCR was carried out by using a RT-PCR kit. IL-6 mRNA expression was examined as described previously (Obara et al., 2001). For analysis of human IL-6 mRNA, the sense primer (5'-AAA TTC GGT ACA TCC TCG AC-3' of human IL-6 cDNA) and the antisense primer (5'-CAG GAA CTG GAT CAG GAC TT-3'), which were complementary to conserved regions of cDNA from human IL-6, were used. For analysis of mouse IL-6 mRNA, the sense primer (5'-CAA GAG ACT TCC ATC CAG TTG C-3') and the antisense primer (5'-TTG CCG AGT AGA TCT CAA AGT GAC-3') were used. The human IL-6 cDNA of 295 base pairs was amplified from 26 to 30 cycles (95°C for 60 s, 56°C for 60 s, and 74°C for 60 s), and mouse IL-6 cDNA of 614 base pairs was amplified 28 cycles (94°C for 30 s, 57°C for 60 s, and 72°C for 60 s). Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) transcripts of 700 base pairs were used as an internal control. The number of cycles that yielded a quantitative amount of product was determined in preliminary experiments. The densities of the bands corresponding to IL-6 and G3PDH were analyzed by densitometry (NIH Image software), and the data were expressed as a ratio to G3PDH.

Immunostaining. Mouse astrocytes were fixed with 4% paraformaldehyde and stained with anti-GFAP antibody (1:10 dilution) and fluorescein isothiocyanate-anti-mouse antibody (1:80 dilution).

Plasmids, Transfection, and Reporter Gene Assay. Plasmids containing IL-6 promoter (wild type or CRE mutated) linked to luciferase gene (Funakoshi et al., 1999; Tokunou et al., 2001) were kindly given by Dr. Toshihiro Ichiki (Kyushu University, Fukuoka, Japan). Human mitogen- and stress-activated protein kinase 1 (MSK1) wild-type and the kinase-dead mutant (MSK1 D195A/D565A) (Arthur et al., 2004) were from Dr. Soren Impey (Vollum Institute, Oregon Health Sciences University, Portland, OR). A plasmid encoding PKI was from Dr. Philip Stork (Vollum Institute, Oregon Health Sciences University). Plasmids encoding constitutively active mutants of G-proteins (G α_q Q209L, G α_{12} Q231L, and G α_{13} Q226L) were kindly provided by the laboratory of John Scott (Vollum Institute, Oregon Health Sciences University). For transfection, 1321N1 cells were seeded onto 24-well plates at 1 \times 10⁵ (cells/well). On the next day, plasmids (0.6–1.2 μ g/well) and FuGENE 6

(0.75–1.8 $\mu\text{l/well}$) were mixed gently in 25 μl of DMEM and added to culture plates. For luciferase assay, media were replaced by serum-free media 24 h after transfection. The cells were incubated with the indicated drugs at 37°C for 6 h on the following day. Luciferase assay was performed by using dual luciferase assay kit (Promega, Madison, WI) according to its instruction. *Renilla reniformis* luciferase (phRL-TK, Promega) activity was measured for normalizing the transfection efficiency. The luciferase activity was measured using a luminometer (GENE LIGHT 55; Microtech Niton, Funabashi, Japan).

cAMP Assay. Intracellular cAMP concentration was measured as follows. 1321N1 cells on a 24-well plate were labeled with 2 $\mu\text{Ci/ml}$ [^3H]adenine (23 Ci/mmol; Amersham Biosciences) in the growth medium for 3 h. The labeled cells were washed twice with DMEM-HEPES (20 mM, pH 7.4). The cells were incubated for 10 min with U46619 (or with SQ29548) in DMEM-HEPES containing phosphodiesterase inhibitor Ro20-1724 (100 μM). After aspirating the incubation buffer, the reactions were stopped by adding 0.4 ml of 2.5% HClO_4 . Acid extracts were mixed with 1/10 volume of 4.2 M KOH to neutralize and deposit potassium perchlorate. [^3H]cAMP in the clear supernatant was separated by Dowex 50W-X8 (Bio-Rad, Hercules, CA)/alumina (Merck Biosciences, Darmstadt, Germany) double columns and determined by liquid scintillation counting. An increase in [^3H]cAMP was expressed as the -fold over the control (no drug).

Adenoviral Infection. Adenoviruses encoding carboxyl-terminal regions of $\text{G}\alpha_q$ ($\text{G}\alpha_q$ -ct, amino acids 305–359), $\text{G}\alpha_{12}$ ($\text{G}\alpha_{12}$ -ct, amino acids 325–379), and $\text{G}\alpha_{13}$ ($\text{G}\alpha_{13}$ -ct, amino acids 322–378) were created in our laboratory. The expressions of those genes are regulated by CMV promoter, and their products putatively block G-protein coupling with their corresponding receptors (Arai et al., 2003). Each adenoviral vector contains both G-protein-ct and GFP that is upstream of the G-protein-ct, and each entity is under the control of CMV promoter. 1321N1 cells were infected with the indicated virus at 75 m.o.i. for 1 day. The medium was then replaced with serum-free medium on the next day, and the cells were cultured overnight. At the time of drug incubation, more than 90% of the cells were GFP-positive.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting. Samples used for immunoblotting were prepared as follows. 1321N1 cells were seeded onto six-well plates at a density of 1×10^6 (cells/well). The cells were cultured overnight in serum-free DMEM, and then drugs were added for various periods of time. The incubation medium was aspirated after the reaction, and the cells were dissolved in Laemmli sample buffer (final concentration: 75 mM Tris-HCl, 2% SDS, 15% glycerol, 3% 2-mercaptoethanol, pH 6.8) and boiled at 95°C for 5 min.

Electrophoresis was performed on 11% acrylamide gels. Proteins were transferred electrically from the gel onto a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA) by the semidry blotting method. The blots were blocked for 40 min with 5% skim milk in Tris-buffered saline supplemented with 0.1% Tween 20 and incubated with primary antibodies [anti-phospho-ERK (Thr202/Tyr204) antibody, anti-ERK2 antibody, anti-phospho-p38 MAPK (Thr180/Tyr182) antibody, anti-p38 MAPK antibody, anti-phospho-CREB (Ser133) antibody, or anti-CREB antibody (1:1000 dilution)] overnight at 4°C. The blots were washed several times and then incubated at 25°C for 2 h with a 1:3000 dilution of secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody). Blots were developed using an enhanced chemiluminescence assay kit (Amersham Biosciences) and visualized by chemiluminescence on Hyperfilm ECL. The densities of the bands corresponding to CREB, ERK, and p38 MAPK were analyzed by densitometry (NIH Image), and the data were expressed as a ratio to their total proteins.

Statistical Methods. Data were expressed as the mean values \pm S.E.M., and the significant differences were analyzed by Student's *t* test or Tukey's method for multiple comparisons.

Results

U46619 Enhances IL-6 Production in 1321N1 Cells.

We investigated the biosynthesis of IL-6. 1321N1 cells were stimulated with the TP agonist, U46619, and then we

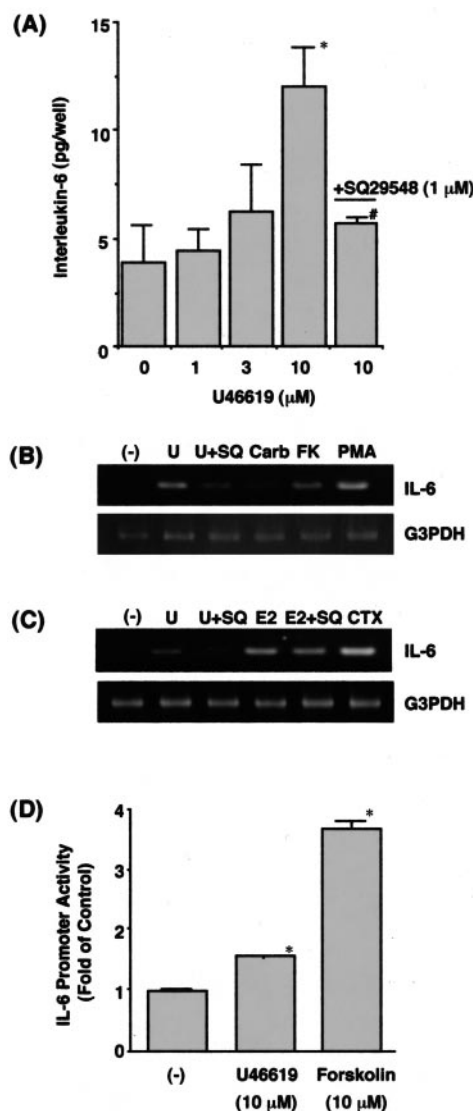


Fig. 1. Induction of IL-6 by U46619 in 1321N1 cells. A, effect of U46619 on the secretion of IL-6 from 1321N1 cells. After incubation with the indicated concentrations of U46619 for 24 h, IL-6 released from 1321N1 cells was measured by ELISA. Values are the means \pm S.E.M. of three determinations. U46619 (10 μM) significantly increased IL-6 secretion compared with the corresponding control (*, $P < 0.05$), and this effect was blocked by 1 μM SQ29548 (#, $P < 0.05$). B, effect of U46619 on IL-6 gene expression in 1321N1 cells. 1321N1 cells were incubated with 10 μM U46619 (U), U46619 + 1 μM SQ29548 (SQ), 10 μM carbachol (Carb), 10 μM forskolin (FK), or 100 nM PMA for 4 h in serum-free condition, and then RT-PCR was performed as described under *Materials and Methods*. C, specificity of U46619 and SQ29548 to TPs. 1321N1 cells were incubated with 10 μM U46619 (U), U46619 + 1 μM SQ29548 (SQ), 1 μM prostaglandin E_2 (E2), prostaglandin E_2 + SQ29548, or 100 $\mu\text{g/ml}$ cholera toxin (CTX) for 4 h in serum-free condition, and then RT-PCR was performed as described under *Materials and Methods*. D, effect of U46619 on IL-6 promoter activity in 1321N1 cells. 1321N1 cells were transfected with plasmid encoding IL-6 promoter-luciferase gene. After overnight serum starvation, the cells were incubated with 10 μM U46619 or 10 μM forskolin for 6 h. Luciferase activity was measured as described under *Materials and Methods*. Values are the means \pm S.E.M. of three determinations. U46619 (10 μM) and 10 μM forskolin significantly increased IL-6 promoter activity compared with the corresponding control (*, $P < 0.05$).

examined IL-6 secretion by sandwich-ELISA (Fig. 1A). U46619 significantly increased IL-6 secretion from 1321N1 cells in a concentration-dependent manner that was inhibited by TP antagonist SQ29548. Adenylyl cyclase activator, forskolin, and cAMP analog dbcAMP also promoted IL-6 secretion by 1.6- and 2.9-fold, respectively (data not shown). U46619 (10 μ M) also enhanced IL-6 gene expression (5.8-fold), and this effect was abolished by 1 μ M SQ29548 again (Fig. 1B). Carbachol (10 μ M) did not affect IL-6 gene expression. Forskolin and a PKC activator, PMA, enhanced IL-6 expression 4.5- and 7.0-fold in the same condition, respectively (Fig. 1B). U46619 and SQ29548 have low affinity to other prostanoid receptors, such as the prostaglandin E₂ receptor subtype EP₄ (Kiriya et al., 1997), and it is also known that prostaglandin E₂ induces IL-6 release in U373 MG human astrocytoma cells (Fiebich et al., 2001). To avoid the possibility that the effect of U46619 on IL-6 induction is mediated by EPs, we tested prostaglandin E₂. U46619-induced IL-6 expression was completely blocked by SQ29548 (3% U46619 alone), whereas most of the effect of prostaglandin E₂ was not blocked by SQ29548 (82% prostaglandin E₂ alone) (Fig. 1C). Cholera toxin as a positive control enhanced IL-6 expression 13.2-fold (Fig. 1C). This result indicates that the effect of U46619 is mediated by TP. To examine the IL-6 promoter activity, the plasmid encoding wild-type IL-6 promoter (-1039 ~ +1) linked to luciferase gene was transfected into 1321N1 cells and luciferase activity was measured (Fig. 1D). U46619 (10 μ M) as well as 10 μ M forskolin significantly enhanced IL-6 promoter activity. Furthermore, GFAP-positive cultured mouse astrocytes (Fig. 2A) were stimulated with U46619 to determine whether IL-6 enhancement is 1321N1 cell-specific or not. As expected, 10 μ M U46619 similarly promoted IL-6 gene expression (2.7-fold) in astrocytes as dbcAMP (2.1-fold), and the effect of U46619 was mostly reversed by SQ29548 (12% U46619 alone) (Fig. 2B). Taken together, these results suggest that U46619 enhances the secretion of IL-6 from 1321N1 cells accompanied by an increase in IL-6 gene expression.

CRE Regulates the IL-6 Gene Expression by U46619 in 1321N1 Cells. IL-6 gene expression is regulated by multiple transcription factors, including CREB, activator pro-

tein-1 (AP-1), nuclear factor- κ B (NF- κ B), CCAAT enhancer-binding protein- β , and glucocorticoid receptor in complex ways (Obara and Nakahata, 2002). Because CREB activation by U46619 has been reported (Muja et al., 2001), we determined whether CREB is involved in the U46619-induced IL-6 gene expression. CRE activity was measured by reporter gene assay. pCRE-luciferase plasmid was transfected into 1321N1 cells, and then newly induced luciferase activity was regarded as the CREB-transcriptional activity. U46619 (10 μ M) and 10 μ M forskolin significantly increased CRE activity (Fig. 3A). To subsequently confirm the specificity of CRE in enhanced IL-6 gene expression, we used the IL-6 promoter-luciferase plasmid harboring a mutant CRE in the IL-6

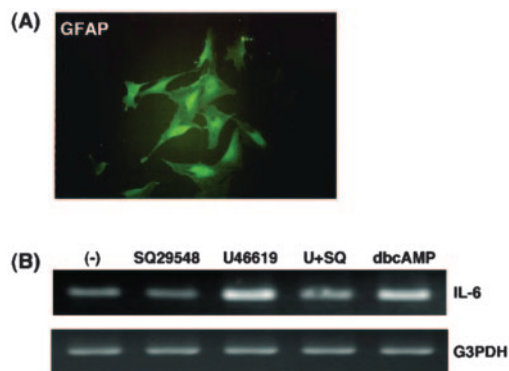


Fig. 2. Induction of IL-6 by U46619 in mouse astrocytes. A, identification of astrocytes. Mouse astrocytes prepared from mouse brains were identified by immunostaining with anti-GFAP antibody. B, IL-6 induction by U46619. Mouse astrocytes were incubated with 1 μ M SQ29548 (SQ), 10 μ M U46619 (U), U46619 + SQ29548 or 0.5 mM dbcAMP for 4 h in the serum-free condition, and then RT-PCR was performed.

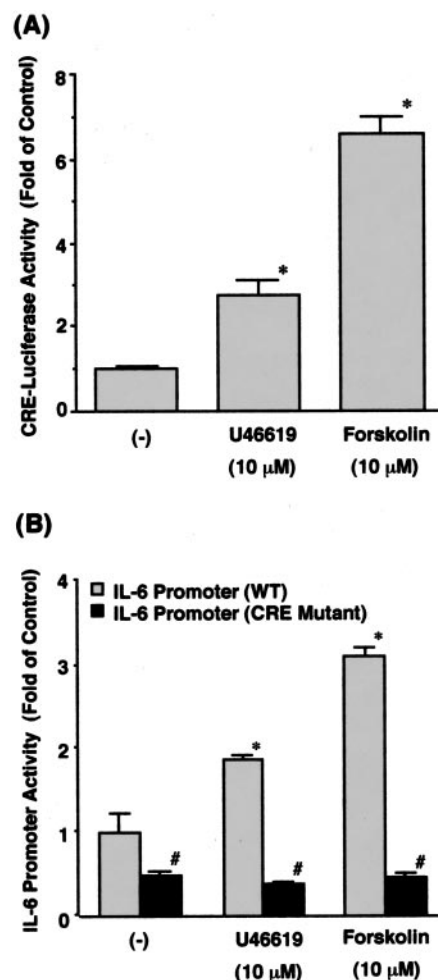


Fig. 3. Involvement of CRE in IL-6 induction by U46619 in 1321N1 cells. A, increase in CRE activity by U46619. After 1321N1 cells were transfected with CRE-luciferase reporter gene plasmid, they were stimulated with 10 μ M U46619 or 10 μ M forskolin for 6 h in the serum-free condition. After the incubation, luciferase assay was performed. Values represent the means \pm S.E.M for three determinations. U46619 (10 μ M) and 10 μ M forskolin significantly increased the CRE activity (*, $P < 0.05$). B, CRE is essential for IL-6 expression. After 1321N1 cells were transfected with plasmid encoding wild-type (WT) IL-6 promoter-luciferase gene or its CRE mutant, they were stimulated with 10 μ M U46619 or 10 μ M forskolin for 6 h in the serum-free condition. After the incubation, luciferase assay was performed. Values represent the means \pm S.E.M for three determinations. U46619 (10 μ M) and 10 μ M forskolin significantly increased the IL-6 promoter activity (*, $P < 0.05$), and mutation at CRE significantly suppressed both activities (#, $P < 0.05$).

promoter region (from ATGACGTCA to ATCGATCCA). The effect of U46619 on IL-6 promoter activity was completely abolished by mutation of CRE as in the case of forskolin (Fig. 3B). These results indicate that CRE activation is essential for U46619-induced IL-6 gene expression in 1321N1 cells.

PKA and p38 MAPK/MSK Pathways Are Involved in CREB Activation and IL-6 Gene Expression by U46619 in 1321N1 Cells. It has been shown that CREB activity is regulated by phosphorylation at Ser133, where various kinases have been proposed to phosphorylate, including pro-

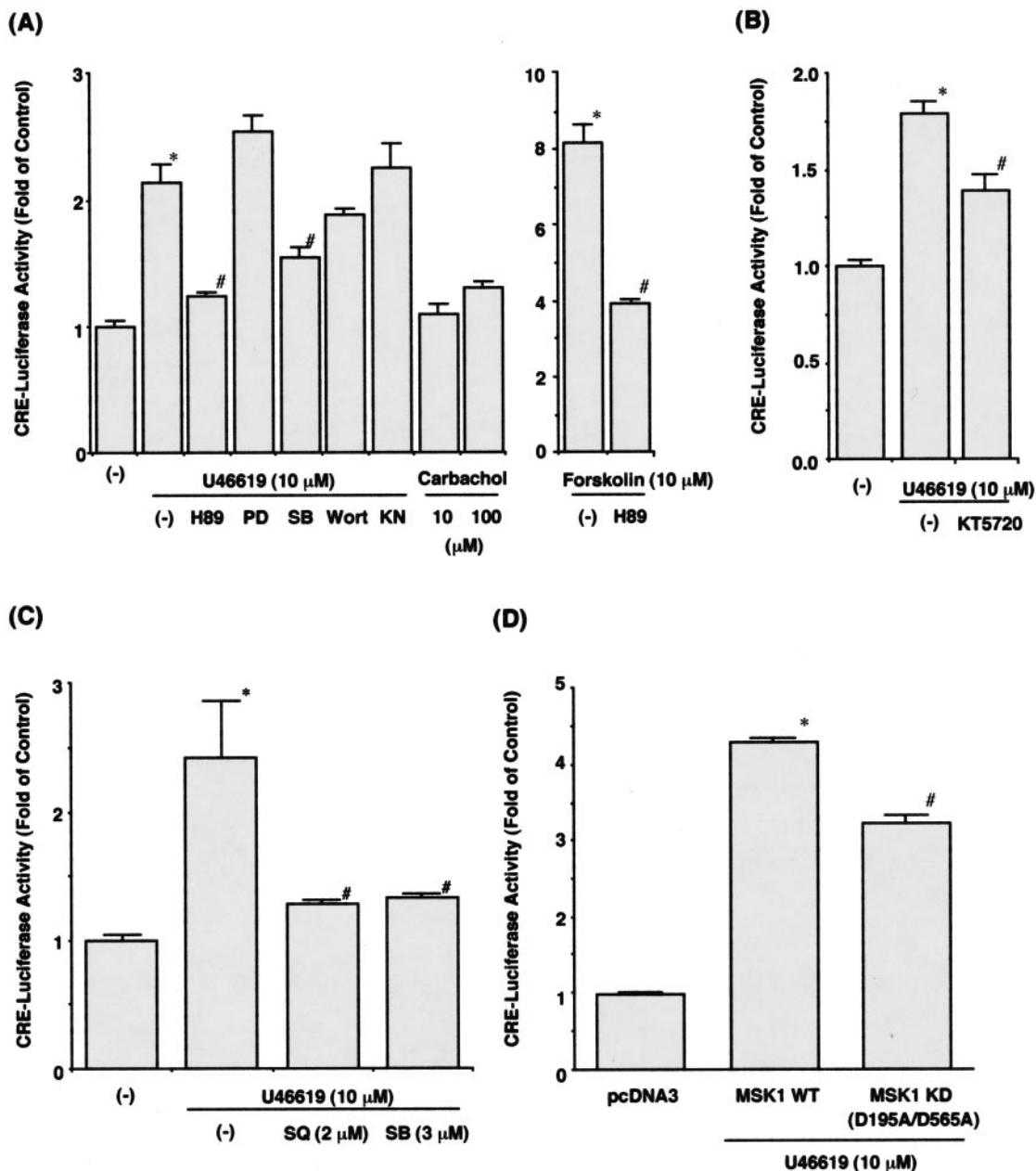


Fig. 4. Involvements of PKA and p38 MAPK in CRE activation by U46619 in 1321N1 cells. A, effects of pharmacological inhibitors on U46619-induced CRE activation. After 1321N1 cells were transfected with CRE-luciferase reporter gene plasmid, they were pretreated with 10 μ M H89, 30 μ M PD98059 (PD), 30 μ M SB203580 (SB), 1 μ M wortmannin (Wort), or 10 μ M KN62 (KN) for 15 min. They were then stimulated with 10 μ M U46619, 10 or 100 μ M carbachol, or 10 μ M forskolin for 6 h in the serum-free condition. After incubation, luciferase assay was performed. Values represent the means \pm S.E.M for three determinations. U46619 (10 μ M) and 10 μ M forskolin significantly increased the CRE activity (*, $P < 0.05$), which was inhibited by 10 μ M H89 and 30 μ M SB203580 (#, $P < 0.05$). B, effect of KT5720 on CRE activated by U46619. 1321N1 cells were pretreated with 2 μ M KT5720 and then stimulated with 10 μ M U46619 for 6 h as performed above. Values represent the means \pm S.E.M for three determinations. U46619 significantly increased the CRE activity (*, $P < 0.05$), which was blocked by 2 μ M KT5720 (#, $P < 0.05$). C, effects of SQ29548 and SB203580 on CRE activated by U46619. 1321N1 cells were pretreated with 2 μ M SQ29548 or 3 μ M SB203580 and then stimulated with 10 μ M U46619 for 6 h as performed above. Values represent the means \pm S.E.M for three determinations. U46619 significantly increased the CRE activity (*, $P < 0.05$), which was blocked by 2 μ M SQ29548 and 3 μ M SB203580 (#, $P < 0.05$). D, involvement of MSK in CRE activated by U46619. MSK1 wild-type (WT) or the kinase-dead mutant (KD) (D195A/D565A) was overexpressed in 1321N1 cells, and then the cells were stimulated with 10 μ M U46619 for 6 h as performed above. Values represent the means \pm S.E.M for three determinations. U46619 significantly increased the CRE activity in the cells overexpressing MSK1 wild type (*, $P < 0.05$). The effect was blocked by mutations in two kinase domains (#, $P < 0.05$).

tein kinase A (PKA), Akt (or protein kinase B), calmodulin-dependent kinases (CaMKs), ribosomal S6 kinases (RSKs), and MSKs (Lonze and Ginty, 2002). Akt is a downstream effector of phosphoinositide 3-kinase, and RSKs and MSKs can be activated by ERK1/2, ERK5, or p38 MAPK. To investigate the U46619 signaling to CREB in detail, pharmacological tools were used to identify the CREB kinase. 1321N1 cells were pretreated with 10 μ M H89 (PKA inhibitor), 30 μ M PD98059 (MEK inhibitor), 30 μ M SB203580 (p38 MAPK inhibitor), 1 μ M wortmannin (phosphoinositide 3-kinase inhibitor), or 10 μ M KN62 (CaMK inhibitor) for 15 min, and then they were stimulated with 10 μ M U46619 for 6 h (Fig. 4A). The U46619-induced CRE activation was significantly blocked by H89 and SB203580. Carbachol (10 or 100 μ M) did not increase the CRE activity. Because H89 has been reported to inhibit MSK as well as PKA (Davies et al., 2000), another PKA inhibitor, KT5720, was tested. KT5720 (2 μ M) also inhibited the CRE activity significantly (Fig. 4B). Increase in CRE activity by U46619 was also blocked by 2 μ M SQ29548 and a lower concentration of 3 μ M SB203580 (Fig. 4C). Furthermore, MSK 1 wild-type and the kinase-dead mutant (D195A and D565A) were overexpressed in 1321N1 cells, and CRE activity was measured (Fig. 4D). The MSK1 kinase-dead mutant partially but significantly attenuated the ability of U46619 to increase CRE activity. CREB family members, including CREB, activating transcription factor 1, and CRE modulator, binds to CRE. To investigate whether CREB is involved in this process, CREB phosphorylation at Ser133 was monitored. This phosphorylation site can be regarded as an index of CREB activation. The CREB phosphorylation by U46619 via TPs was observed (Fig. 5A), confirming that CREB is activated by U46619. This phosphorylation was completely blocked by 10 μ M H89, 30 μ M SB203580, and 2 μ M KT5720 again (Fig. 5B). These results suggest that PKA and p38 MAPK/MSK pathways mediate CREB activation by

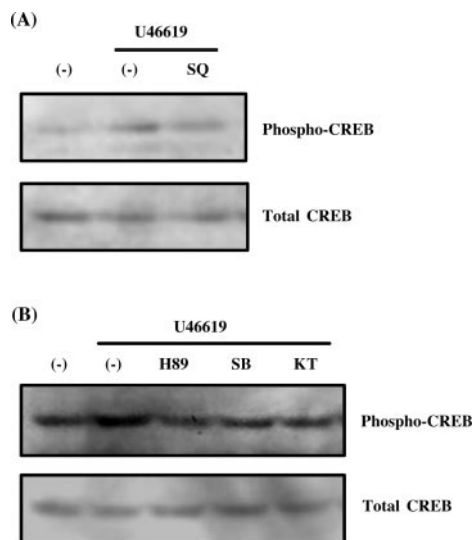


Fig. 5. CREB phosphorylation by U46619 in 1321N1 cells. A, CREB at Ser133 is phosphorylated by U46619 via TPs. 1321N1 cells were stimulated with 10 μ M U46619 for 10 min in the presence or absence of 2 μ M SQ29548 (SQ) followed by Western blotting using antibodies against phospho-CREB and CREB. B, CREB at Ser133 is phosphorylated by U46619 via PKA and p38 MAPK. 1321N1 cells were stimulated with 10 μ M U46619 for 10 min in the presence or absence of 10 μ M H89, 30 μ M SB203580 (SB), and 2 μ M KT5720 (KT) followed by Western blotting using antibodies against phospho-CREB and CREB.

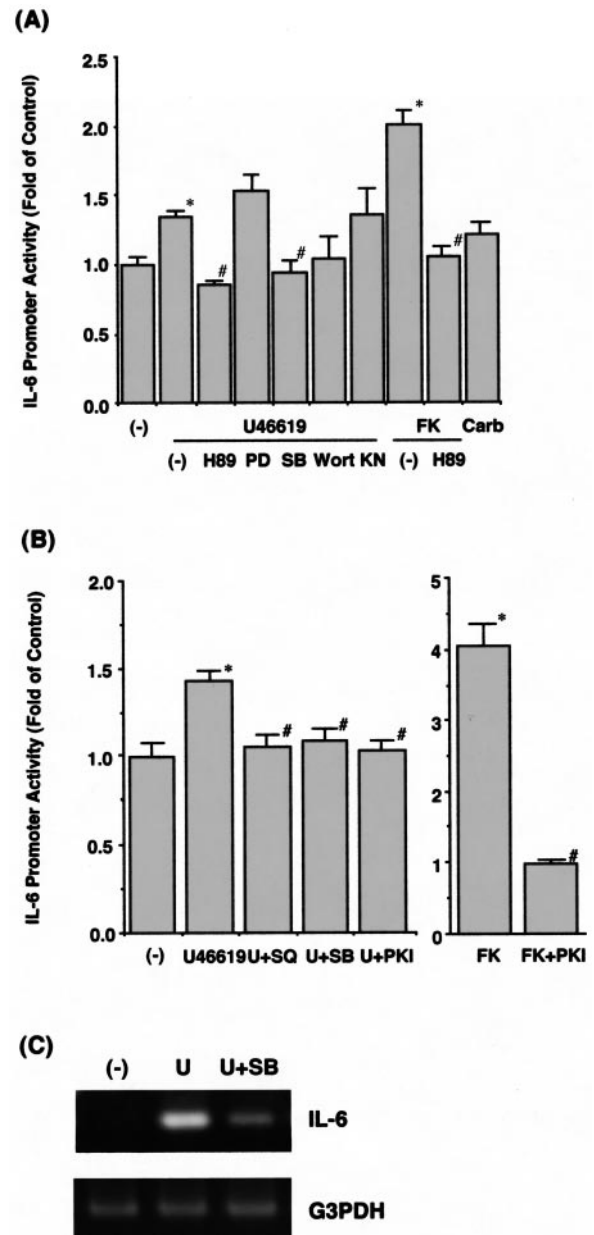


Fig. 6. Involvements of PKA and p38 MAPK in IL-6 expression in 1321N1 cells. A, effects of several kinase inhibitors on IL-6 promoter activity. After 1321N1 cells were transfected with plasmid encoding IL-6 promoter-luciferase reporter gene plasmid, the cells were pretreated with 10 μ M H89, 30 μ M PD98059 (PD), 30 μ M SB203580 (SB), 1 μ M wortmannin (Wort), or 10 μ M KN62 (KN) for 15 min. They were then stimulated with 10 μ M U46619, 10 μ M carbachol (Carb), or 10 μ M forskolin (FK) for 6 h in the serum-free condition. After the incubation, luciferase assay was performed. Values represent the means \pm S.E.M for three determinations. U46619 (10 μ M) and 10 μ M forskolin significantly increased the IL-6 promoter activity (*, $P < 0.05$), which was inhibited by 10 μ M H89 and 30 μ M SB203580 (#, $P < 0.05$). B, involvements of TPs, p38 MAPK, and PKA in U46619-activated IL-6 promoter activity. After 1321N1 cells were cotransfected with plasmid encoding IL-6 promoter-luciferase reporter gene plasmid and PKI, the cells were pretreated with 2 μ M SQ29548 (SQ) or 3 μ M SB203580 (SB) for 15 min. They were then stimulated with 10 μ M U46619 or 10 μ M forskolin (FK) for 6 h in the serum-free condition. After the incubation, luciferase assay was performed. Values represent the means \pm S.E.M for three determinations. U46619 (10 μ M) and 10 μ M forskolin significantly increased the IL-6 promoter activity (*, $P < 0.05$), which was inhibited by 2 μ M SQ29548, 3 μ M SB203580, and PKI (#, $P < 0.05$). C, inhibitory effect of SB203580 on IL-6 gene expression. 1321N1 cells were incubated with 10 μ M U46619 (U) in the presence or absence of 30 μ M SB203580 (SB) for 4 h in the serum-free condition. RT-PCR was then performed.

U46619. The effects of these kinase inhibitors on IL-6 gene expression were investigated next. H89 (10 μ M) and 30 μ M SB203580 decreased IL-6 promoter activity accelerated by 10 μ M U46619 (Fig. 6A), similar to the inhibition of CRE activity shown in Fig. 4A. The effect of U46619 was also significantly inhibited by 2 μ M SQ29548 and 3 μ M SB203580, overexpressing a physiological PKA pseudosubstrate peptide, PKI (Fig. 6B). Furthermore, when 1321N1 cells were pretreated with 30 μ M SB203580, IL-6 gene expression induced by 10 μ M U46619 was largely suppressed (21% U46619 alone), as determined by RT-PCR (Fig. 6C). Because the involvement of p38 MAPK in TP-mediated IL-6 expression was suggested, we examined whether p38 MAPK was activated by U46619. When 1321N1 cells were incubated with 10 μ M U46619 for 5 to 20 min, transient phosphorylation of ERK1/2 was observed (2.2-fold at 5 min, 1.9-fold at 10 min, and 0.97-fold at 20 min) (Fig. 7). In addition to ERKs, p38 MAPK phosphorylation was induced by 10 μ M U46619 for 5 to 20 min (1.8-fold at 5 min, 2.1-fold at 10 min, and 2.8-fold at 20 min). CREB phosphorylation at Ser133 in the same lysates was also monitored. As expected, U46619 induced CREB phosphorylation correlated with p38 MAPK phosphorylation (5.6-fold at 5 min, 9.1-fold at 10 min, and

2.9-fold at 20 min) (Fig. 7). G_{α_s} coupling to TPs has not currently been reported, although an intracellular cAMP concentration by TP agonist is elevated in many cell types. We measured cAMP level after U46619 treatment (Fig. 8). The β -adrenergic receptor agonist isoproterenol (10 μ M) strongly elevated the cAMP level. Compared with isoproterenol, the effect of U46619 on cAMP accumulation was much weaker, but the significant cAMP increase via TPs was observed. Taken together, these results suggest that PKA and p38 MAPK/MSK pathways are involved in CREB activation and IL-6 gene expression induced by U46619 in 1321N1 cells.

G_{α_q} and $G_{\alpha_{13}}$ Mediate TXA_2 Signaling to IL-6 Expression. TPs couple with various kinds of G-proteins, such as G_{α_q} , $G_{\alpha_{12}}$, and $G_{\alpha_{13}}$ (Huang et al., 2004). We have shown that TPs associate with at least G_{α_q} and $G_{\alpha_{12}}$ in 1321N1 cells (Honma et al., 1998). To determine which G-proteins are involved in the signaling pathway leading to IL-6 production, we took an adenoviral approach to interfere with endogenous G-protein functions. The products from adenoviruses that encode the C-terminal regions of G_{α_q} , $G_{\alpha_{12}}$, or $G_{\alpha_{13}}$ inhibited the coupling among these G-proteins and their associated receptors (Arai et al., 2003). GFP gene was introduced to monitor the infection efficiency of those G-protein-interfering mutants. 1321N1 cells infected with those viruses were stimulated with 10 μ M U46619 for 4 h, and then RT-PCR was carried out. IL-6 gene expression was promoted with U46619 treatment in 1321N1 cells infected with control virus, and the enhancement was blocked by overexpressing C-terminal regions of G_{α_q} or $G_{\alpha_{13}}$ [45 and 53% U46619 (GFP), respectively] but not by $G_{\alpha_{12}}$ (Fig. 9A). Furthermore, to determine whether these G-proteins can affect IL-6 production, the cells were transfected with constitutively active mutants ($G_{\alpha_q}Q209L$, $G_{\alpha_{12}}Q231L$, and $G_{\alpha_{13}}Q226L$). $G_{\alpha_q}Q209L$ and $G_{\alpha_{13}}Q226L$ promoted IL-6 gene expression 15- and 7.9-fold, respectively, determined by RT-PCR, whereas $G_{\alpha_{12}}Q231L$ marginally promoted it (2.6-fold) (Fig. 9B). In addition, these active mutants could significantly enhance both CRE and IL-6 promoter activities (Fig. 9C). Taken together, the results suggest that G_{α_q} and $G_{\alpha_{13}}$ are necessary and sufficient for U46619-induced IL-6 gene expression.

Discussion

In the present study, we showed that U46619 promoted IL-6 biosynthesis in 1321N1 human astrocytoma cells and

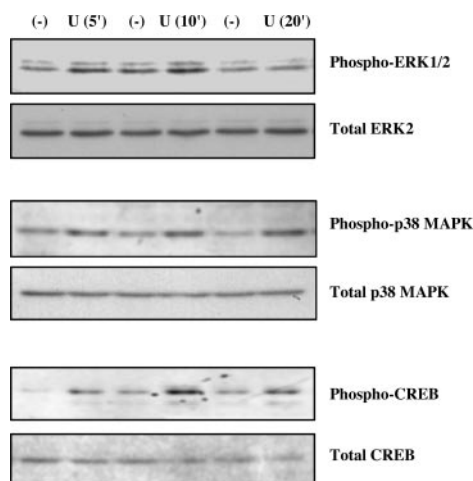


Fig. 7. Effect of U46619 on phosphorylation of ERK1/2, p38 MAPK, or CREB in 1321N1 cells. 1321N1 cells were stimulated with 10 μ M U46619 (U) for 5, 10, or 20 min followed by Western blotting using antibodies against phospho-ERK, ERK2, phospho-p38 MAPK, p38 MAPK, phospho-CREB, and CREB.

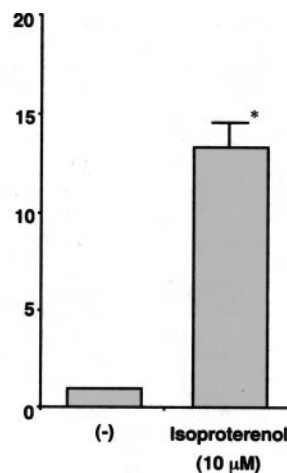
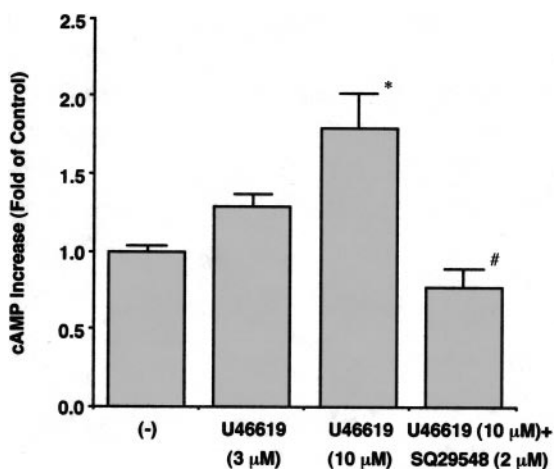


Fig. 8. U46619 promotes cAMP accumulation in 1321N1 cells. 1321N1 cells were labeled with 2 μ Ci/ml [3 H]adenine for 3 h and then stimulated with 3 or 10 μ M U46619, 10 μ M U46619 + 2 μ M SQ29548, or 10 μ M isoproterenol for 10 min in the presence of phosphodiesterase inhibitor, Ro20-1724 (100 μ M). Values represent the means \pm S.E.M for three determinations. U46619 (10 μ M) and 10 μ M isoproterenol significantly accumulated cAMP (*, $P < 0.05$), which was blocked by SQ29548 (#, $P < 0.05$).

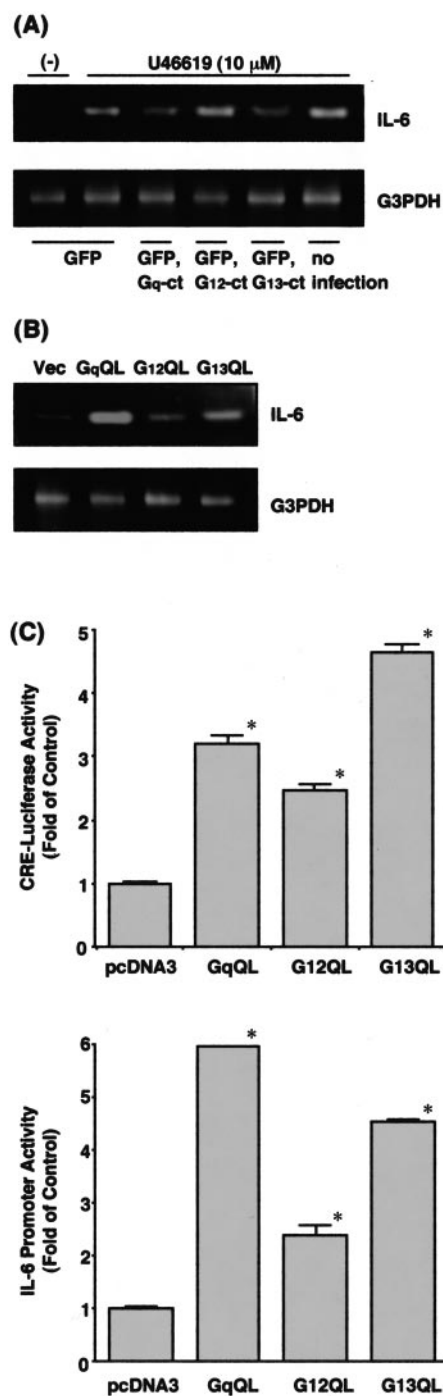


Fig. 9. Involvements of $G\alpha_q$ and $G\alpha_{13}$ in the U46619-enhanced IL-6 production in 1321N1 cells. **A**, inhibition of IL-6 gene expression by C-terminal peptides of $G\alpha$ proteins. 1321N1 cells were infected with adenovirus encoding GFP, GFP/ $G\alpha_q$ -ct, GFP/ $G\alpha_{12}$ -ct, or GFP/ $G\alpha_{13}$ -ct at 75 m.o.i. for a day, and then the cells were serum-starved overnight. The cells were stimulated with 10 μM U46619 for 4 h, and then RT-PCR was performed to examine IL-6 gene expression. **B**, $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_{13}$ are sufficient for IL-6 gene expression. 1321N1 cells were transfected with constitutively active mutants of $G\alpha_q$, $G\alpha_{12}$, or $G\alpha_{13}$ ($G\alpha_q$ QL, $G\alpha_{12}$ QL, and $G\alpha_{13}$ QL, respectively). The cells were serum-starved overnight 24 h after transfection, and then RT-PCR was performed to examine basal IL-6 gene expression. **C**, $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_{13}$ are sufficient for activation of CRE and IL-6 promoter. 1321N1 cells were cotransfected with constitutively active mutants of $G\alpha_q$, $G\alpha_{12}$, or $G\alpha_{13}$ ($G\alpha_q$ QL, $G\alpha_{12}$ QL, and $G\alpha_{13}$ QL, respectively) and pCRE- or pIL-6-luciferase, and then luciferase activity was measured. Values represent the means \pm S.E.M. for three determinations. $G\alpha_q$ QL, $G\alpha_{12}$ QL, and $G\alpha_{13}$ QL significantly increased the CRE and IL-6 promoter activity (*, $P < 0.05$).

GFAP-positive mouse astrocytes. In addition, U46619 increased CREB activity that was accompanied by its phosphorylation at Ser133 by PKA and p38 MAPK/MSK, and this CREB activation is essential for IL-6 induction in 1321N1 cells (Fig. 10). This is the first report showing TP-mediated IL-6 production in glial cells.

The amount of arachidonic acid metabolites are increased dramatically after brain injury, viral infection, or inflammation. These metabolites, including TXA₂, are assumed to play important roles in the CNS under such pathologic conditions. In fact, astrocytes and microglia have the ability to release TXA₂ after activation by bacterial lipopolysaccharide and ATP (Pearce et al., 1989; Bruner and Murphy, 1993; Minghetti and Levi, 1995). Thus, the elevation of adenine nucleotide concentration or microglial activation after CNS injury or infection may cause the secretion of TXA₂, leading to activation of TPs on glial cells via autocrine or juxacrine mechanisms. Therefore, a clarification of the physiological roles of TPs in astrocytes will help in identifying therapeutic targets in various CNS disorders.

IL-6 is a pleiotropic factor involved in the regulation of various inflammatory and endocrine functions in both central and periphery nervous systems. In the CNS, although various cell types can produce IL-6, astrocytes play a dominant role in IL-6 production (Gruol and Nelson, 1997). Under normal physiological conditions, IL-6 expression levels in CNS remain low, whereas IL-6 is up-regulated upon stress, diseases, inflammation, or injury. To date, it has been shown that numerous physiological receptor agonists or pharmacological reagents influence IL-6 production (Sehgal, 1992; Van Wagoner and Benveniste, 1999). Cytokines and proinflammatory factors, including lipopolysaccharide, IL-1 β , tumor necrosis factor- α , and prostaglandin E₂ are known as IL-6 inducers and play important roles in CNS diseases. In addition, various kinds of neurotransmitters and neuropeptides, including catecholamines, adenosine, serotonin, histamine, substance P, calcitonin, vasoactive intestinal peptide, and pituitary adenylate cyclase-activating polypeptide, also influence IL-6 production. Abnormal physiological condition often changes the induction of IL-6, such as hypoxia/reoxygenation, generation of reactive oxygen species, viral infections,

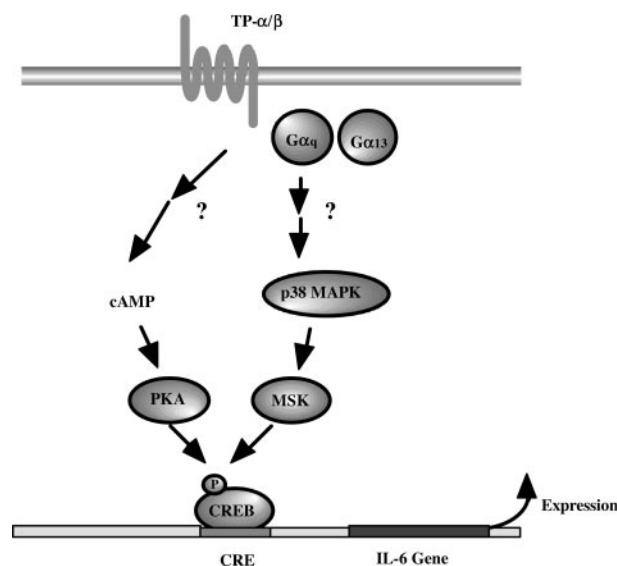


Fig. 10. Diagram of putative signaling pathway of TXA₂.

and irradiation. It is not surprising that compounds that increase cyclic AMP concentration, such as cholera toxin, cyclic AMP analogs, and forskolin, also lead to an induction of IL-6 in astrocytes (Norris et al., 1994). Furthermore, phorbol ester and Ca^{2+} ionophore, A23187, induced IL-6 production and both of these reagents caused synergistic production, suggesting that PKC and Ca^{2+} signaling are responsible for IL-6 production in rat astrocytes (Norris et al., 1994). Fiebich et al. (1995) showed that ceramide induced IL-6 gene expression in U373-MG human astrocytoma cells. In terms of transcription factors, IL-6 promoter contains various consensus sequences of transcription factors, indicating that multiple transcription factors influence its gene expression. For example, CREB, AP-1, NF- κ B, CCAAT enhancer-binding protein- β (or NF-IL6), and glucocorticoid receptor are known as regulators, and stimuli leading to the activation of these transcription factors are assumed to modulate IL-6 gene expression (Sehgal, 1992; Obara and Nakahata, 2002).

In the present study, it was demonstrated that U46619 increased the CRE activity by reporter gene assay, accompanied by the phosphorylation of CREB at Ser133, which is a key regulatory site for its transcription activity (Figs. 3A, 4, 5, and 7). Furthermore, this CREB activation is required for IL-6 gene expression (Fig. 3B). To date, it has been shown that the phosphorylation of this site occurs via multiple kinases, including PKA, CaMK, Akt, RSKs, and MSKs (Lonze and Ginty, 2002). RSKs and MSKs are known as downstream effectors of ERK1/2, ERK5, and p38 MAPK (Lonze and Ginty, 2002). In our study, to clarify the mechanism leading to CREB activation by U46619, various pharmacological tools were tested. U46619-induced CREB activation was significantly blocked by H89, KT5720, and SB203580 (Fig. 4). In addition, MSK1 kinase-dead mutant blocked CRE activity (Fig. 4D), suggesting the involvements of both PKA and p38 MAPK/MSK pathways. Although the mechanism remains unclear, cyclic AMP elevation and PKA activation have been shown in response to TP agonist (Muja et al., 2001; Miggin and Kinsella, 2002). On the other hand, p38 MAPK activation by TXA_2 has been reported in platelets and isolated rat mesenteric resistance arteries (Bolla et al., 2002; Minuz et al., 2002). In the present study, we confirmed the elevation of cAMP and activation of p38 MAPK by U46619 in 1321N1 cells (Figs. 7 and 8). Taken together, this study strongly indicates that PKA and p38 MAPK participate in CREB activation by TXA_2 . However, as mentioned above, IL-6 gene expression is regulated by multiple transcription factors. Although it was demonstrated in this study that CREB activity was critical for IL-6 induction, we do not deny the possibility that other transcription factors, such as AP-1 and NF- κ B, activated by U46619 (Huang et al., 2004), may work cooperatively with CREB.

We demonstrated previously that TPs coupled with the α subunits of at least two heterotrimeric GTP-binding proteins, G_q and G_{12} , in 1321N1 cells (Honma et al., 1998). Phosphoinositide hydrolysis is promoted through stimulation with TP agonists, which causes increase in intracellular Ca^{2+} concentration and PKC activation (Nakahata et al., 1989). In dbcAMP-treated 1321N1 cells, phosphatidylcholine-specific phospholipase C, rather than phosphatidylinositol-specific phospholipase C, is predominantly activated by TP agonist, which causes subsequent ERK activation (Kobayashi et al., 2000). Because carbachol, an agonist of M_3 receptor that

couples to G_q , neither increased CRE activity nor induced IL-6 expression in this study, it was assumed that the other signaling pathway, such as G_{12} or G_{13} , other than G_q -mediated PLC pathway might be essential for U46619-induced IL-6 expression. In fact, although all of the constitutively active G-protein mutants (G_q , G_{12} , and G_{13}) could activate CRE and IL-6 promoter, interfering mutants G_q -ct and G_{13} -ct, but not G_{12} -ct, blocked IL-6 gene expression by TP stimulation (Fig. 6), indicating that TPs use G_q and G_{13} pathways for IL-6 production. Even though G_{12} and G_{13} belong to the same heterotrimeric G-protein family, the functional difference between those two G-proteins remain unclear. Nevertheless, in our study, G_{13} but not G_{12} was essential for TP signaling. Identification of direct downstream effector of G_{13} leading to CRE activation and IL-6 gene expression is necessary.

In vascular smooth muscle cells, thrombin induces IL-6 expression through the CRE, as in the case of TXA_2 (Tokunou et al., 2001). In that report, involvements of ERK, p38 MAPK, and epidermal growth factor receptor (EGFR) are also implicated in thrombin-induced IL-6 production. EGFR transactivation that lies upstream of ERK and p38 MAPK is essential for this thrombin-induced IL-6 induction. EGFR transactivation by TXA_2 , which was probably mediated by either EGF-like ligand release by metalloproteinases or direct EGFR *trans*-phosphorylation by unidentified tyrosine kinases, has been also reported (Gao et al., 2001; Miggin and Kinsella, 2001). Thus, it is possible that this cross-talk between G-protein-coupled receptors and receptor tyrosine kinases is involved in p38 MAPK and CREB activation leading to IL-6 gene expression.

It has been shown that IL-6 acts as a neurotrophic factor as well as cytokine, and numerous studies have demonstrated both beneficial and unfavorable effects of IL-6 in CNS (Van Wagoner and Benveniste, 1999). A variety of *in vitro* and *in vivo* studies provide evidence for the involvement of IL-6 in neuronal survival, differentiation, and regeneration. IL-6 directly affects neurons, whereas it also affects glial cells; i.e., IL-6 promotes proliferation of astrocytes and production of nerve growth factor by autocrine mechanism. In contrast to the benefits above, IL-6 sometimes cause destructive effects. For example, in IL-6 transgenic mouse models, a high level of IL-6 led to neurodegeneration, blood-brain barrier breakdown, abnormal angiogenesis, increased level of complement proteins, and impaired learning. In those mice, it has been demonstrated that i.c.v. injection of lipopolysaccharide resulted in higher level productions of proinflammatory factors, such as tumor necrosis factor- α and IL-1 β . IL-6 overproduction in the CNS may trigger unnecessary inflammatory responses and confusion of neuronal network. Thus, both beneficial and detrimental effects of IL-6 in the CNS have to be considered for the further study.

In conclusion, we showed that TXA_2 analog induced a production of IL-6 mediated via PKA p38 MAPK/CREB pathway through G_{13} and G_{12} in 1321N1 cells. This observation indicates one of physiological roles of TPs in astrocytes and raises a possibility of TP as a therapeutic target in neuronal diseases.

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