Enterovirus 71 Modulates a COX-2/PGE2/cAMP-Dependent Viral Replication in Human Neuroblastoma Cells: Role of the c-Src/EGFR/p42/p44 MAPK/CREB Signaling Pathway

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ABSTRACT
Enterovirus 71 (EV71) has been shown to induce cyclooxygenase-2 (COX-2) expression in human neuroblastoma SK-N-SH cells through the action of MAPKs, NF-κB, and AP-1. On the other hand, the transcription factor CREB has also been implicated in the expression of COX-2 in other cell lines. Here, we report that EV71-induced COX-2 expression and PGE2 production were both inhibited by pretreatment with the PKA inhibitor H89 or by transfection with CREB siRNA. In addition, EV71-induced COX-2 expression and c-Src/EGFR phosphorylation were both attenuated by transfection with c-Src siRNA or pretreatment with the inhibitors of c-Src (PP1) or EGF receptor (EGFR) (AG1478 and EGFR-neutralizing antibody). We also observed that EV71-induced p42/p44 MAPK phosphorylation was decreased following pretreatment with AG1478. Moreover, EV71-induced COX-2 expression was blocked by pretreatment with the p300 inhibitor GR343 or by transfection with p300 siRNA. Using immunoprecipitation and chromatin immunoprecipitation assays, we observed that EV71 stimulated the association of CREB and p300 with the COX-2 promoter region. Notably, we also demonstrated that EV71-induced COX-2 expression and PGE2 production promoted viral replication via cAMP signaling. In summary, this study demonstrates that EV71 activates the c-Src/EGFR/p42/p44 MAPK pathway in human SK-N-SH cell, which leads to the activation of CREB/p300, and stimulates COX-2 expression and PGE2 release. J. Cell. Biochem. 112: 559–570, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: EV71; COX-2; PGE2; EGF RECEPTOR; CREB; NEUROBLASTOMA CELLS

After the eradication of the poliovirus, the enterovirus 71 (EV71) has been regarded as the most important neurotropic enterovirus able to cause acute inflammation of the central nervous system (CNS) such as encephalitis or meningitis [Chen et al., 2007; Liu et al., 2007a]. Cyclooxygenase-2 (COX-2) plays an important role during acute CNS inflammation or following an injury since it is rapidly induced by various stimuli usually implicated with these conditions, including virus infections. The COX-2 enzyme catalyzes the synthesis of prostaglandins (PGs) in various cell types [Hinson and Tyor, 2001; Chen and Reis, 2002]. The expression of COX-2 is regulated by several transcription factors including the cyclic-AMP response element binding protein (CREB), nuclear factor-kappaB (NF-κB), and activator protein-1 (AP-1) [Tsatsanis et al., 2006]. We had previously demonstrated that EV71 induced COX-2 expression via NF-κB and AP-1 in SK-N-SH cells [Tung et al., 2010]. On the other hand, CREB have also been shown to regulate COX-2 expression by binding to the cyclic adenosine 3',5'-monophosphate (cAMP) response element (CRE) present in the COX-2 promoter which harbors the core sequence CGTCA [Desdouets et al., 1995; Ahn et al., 1998; Lonze and Ginty, 2002; Impey et al., 2004; Zhang et al., 2005; White et al., 2006; Klein et al., 2007].

CREB, a member of the bZIP superfamily of transcription factors [Shaywitz and Greenberg, 1999; Lonze and Ginty, 2002], is activated in response to various stimuli such as growth factors, neurotransmitters, stress signals, and other agents that elevate intracellular cAMP or Ca2+ levels. CREB is activated by phosphorylation at Ser333 by a cAMP-dependent protein kinase (PKA) and/or following nuclear translocation of the transducer of regulated CREB activity [Shaywitz...
Ca\textsuperscript{2+} infection has been shown to activate CREB by a pathway involving interferon regulatory factor-1 (IRF-1) and IRF-3. In this case, SV calcium-independent CREB pathway [Liu et al., 2007b]. In addition, a c-Src/EGFR/p42/p44 MAPK/CREB-dependent pathway in SK-N-SH cells have previously demonstrated that COX-2 expression and PGE\textsubscript{2} production induced by EV71 requires the action of MAPKs (p42/p44 MAPK, p38 MAPK, and JNK1/2), thereby linking EV71 infection with the activation of NF-\kappaB and AP-1. However, whether CREB is implicated in COX-2 expression during EV71 infection in human neurons remains unknown.

Previous studies have reported that CREB is activated by the cAMP/PKA pathway [Desdouets et al., 1995; Ahn et al., 1998; Impey et al., 2004; Zhang et al., 2005; White et al., 2006; Klein et al., 2007]. However, several other signaling pathways have been shown to regulate CREB activity. For instance, angiogenins II activates the AT1 receptor which leads to phosphorylation of CREB via Cdc42/PAK1/MKK3/6/p38 MAPK [Pham et al., 2008]. In addition, CREB activation can be stimulated by either transactivation of EGF receptor (EGFR) by angiotensin II or directly by binding of EGF to the EGFR which activates the Ras/Raf/MAPK and the Rac/PAK1/MKK3/6/p44 MAPK pathways. The activated form of CREB binds to COX-2 promoter and activates COX-2 expression [Pham et al., 2008]. Several studies have demonstrated that CREB phosphorylation is mediated through an EGFR-dependent cascade in various cell types [Amorino et al., 2002; Laag et al., 2006; Ozgen et al., 2008]. However, whether activation of EGFR by EV71 infection could lead to COX-2 expression in neurons remains unclear.

In the present study, we observed that CREB was phosphorylated following transactivation of EGFR by c-Src in EV71-infected cells. CREB phosphorylation promoted the binding to p300, recruitment of the resulting complex to the COX-2 promoter, and stimulation of COX-2 expression in EV71-infected cells. These findings suggest that EV71 induces COX-2 expression and PGE\textsubscript{2} production through a c-Src/EGFR/p42/p44 MAPK/CREB-dependent pathway in SK-N-SH cells.

**MATERIALS AND METHODS**

**MATERIALS**

- Anti-COX-2 (sc-19999), anti-p300 (sc-585), anti-p44 (sc-93), anti-c-Src (sc-18), anti-phospho-c-Src (sc-12928-R), anti-EGFR (sc-03), and anti-phospho-EGFR (sc-23420) were purchased from Santa Cruz (Santa Cruz, CA).
- Anti-CREB (Cat. No. 9197), anti-phospho-CREB (Cat. No. 9191), and anti-phospho-p42/p44 MAPK (Cat. No. 9101) were purchased from Cell Signaling (Danvers, MA). GAPDH (Cat. No. MA1-10036) was from Thermo (Worcester, MA).
- AG1478, PP1, U0126, and GR343 were purchased from Biomol (Plymouth Meeting, PA). CREB, p300, and scrambled siRNA were bought from Invitrogen (Carlsbad, CA). The luciferase assay kit was purchased from Promega (Madison, WI). The enzyme immunoassay kit to detect PGE\textsubscript{2} was obtained from Cayman Chemicals (Ann Arbor, MI).

**CELL CULTURE**

Human neuroblastoma SK-N-SH cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA).

**VIRUS PREPARATION AND PURIFICATION**

EV71 were propagated using human embryonic rhabdomyosarcoma (RD) cells, as previously described [Shih et al., 2004]. Briefly, the cells were first grown to 80% confluence in 175-T flasks. The medium was discarded and the cells were washed twice with phosphate-buffered saline (PBS). The cells were then incubated with viral inoculum at 37\(^\circ\)C for 1 h with occasional shaking. The cells were incubated in DMEM supplemented with 2\% FBS until a 90\% cytopathic effect was reached. After initial centrifugation at 2,000 \(g\) for 5 min, the cell pellet was subjected to three freeze–thaw cycles, followed by centrifugation as above in order to release the intracellular virus particles. Viral aliquots were then stored at -80\(^\circ\)C until use.

**PLAQUE ASSAY**

Vero cells (4 \(\times\) 10\(^5\) cells per well) were seeded overnight into a six-well culture plates and were then infected with a 10-fold serial dilution of virus suspension. After a 1-h adsorption, the cells were washed once with PBS and overlaid with 0.3\% agarose in DMEM containing 2\% FBS. After 96 h, the cells were fixed with 10\% formaldehyde and were then stained with 1\% crystal violet solution. The virus titer was quantified as plaque-forming units (PFU; ml of cells/lysate).

**WESTERN BLOT ANALYSIS**

SK-N-SH cells were plated onto 12-well culture plates and were rendered quiescent by incubation in DMEM/F-12 medium containing 2\% FBS for 24 h. Growth–arrested cells were incubated with or without EV71 at 37\(^\circ\)C for the indicated times. Different concentrations of virus were used. When inhibitors were used, they were added 1 h prior to the application of EV71. After incubation, the cells were then rapidly washed with ice-cold PBS, scraped, and collected by centrifugation at 1,000 \(g\) for 10 min. The collected cells were lysed with ice-cold lysis buffer (25 mM Tris–HCl, pH 7.4, 25 mM NaCl, 25 mM NaF, 25 mM sodium pyrophosphate, 1 mM sodium vanadate, 2.5 mM EDTA, 2.5 mM EGTA, 0.05\% (w/v) Triton X–100, 0.5\% (w/v) SDS, 0.5\% (w/v) deoxycholate, 0.5\% (w/v) NP-40, 5 \(\mu\)g/ml leupeptin, 5 \(\mu\)g/ml aprotinin, and 1 mM PMSF). The cell lysates were centrifuged at 45,000 \(g\) for 1 h at 4\(^\circ\)C to produce the whole cell extract. Samples from these supernatant fractions (30 \(\mu\)g proteins) were denatured and subjected to SDS–PAGE using a 10\% (w/v) running gel. Proteins were transferred to nitrocellulose membranes and the membranes were incubated successively at room temperature with 1\% (w/v) BSA in TTBS for 1 h. Membranes were then
incubated overnight at 4°C using either an anti-COX-2, anti-GAPDH, anti-phospho-CREB, anti-CREB, anti-phospho-p42/p44 MAPK, anti-p44, anti-phospho-EGFR, anti-EGFR, anti-phospho-hcSrc, anti-c-Src, or anti-phospho antibody at a dilution of 1:1,000 in TTBS. The membranes were washed with TTBS four times for 5 min each time, incubated with a 1:2,000-dilution of either anti-rabbit or anti-mouse horseradish peroxidase antibody for 1 h. The immunoreactive bands detected by ECL reagents were developed using the Hyperfilm-ECL (Amersham, Buckinghamshire, UK).

CELLULAR AND VIRAL EXTRACTION

Total cellular RNA was isolated from cells treated with EV71 for the indicated time in 10-cm culture dishes using Trizol according to the protocol of the manufacturer. Total viral RNA was extracted from infected cells using a Viral Nucleic Acid Extraction kit (Biomass Scientific, Taipei, Taiwan) according to the manufacturer’s instructions. Total cellular or viral RNA concentration was determined by monitoring absorbance at 260 nm using a spectrophotometer. The cDNA synthesis was performed with 2 μg of total RNA using random hexamers, 1 mM dNTPs, 2 units/μl RNasin, and 10 units/μl Moloney murine leukemia virus reverse transcriptase. The reaction was carried out at 37°C for 60 min.

REVERSE TRANSCRIPTION (RT) PCR ANALYSIS AND REAL-TIME PCR ANALYSIS

The cDNAs encoding β-actin and COX-2 were amplified from 3 to 5 μl of the cDNA reaction mixture using specific gene primers. The primers for β-actin and COX-2 were as follows: β-actin, 5'-TGAGGGTCTACACCTGTGCCCTACTA-3' (sense) and 5'-CTAGAAGCATTGTGGGAGCACTG-3' (antisense); and COX-2, 5'-TTCAAGAGATTGTGGGAAAATTGCT-3' (sense) and 5'-AGATCATCTCGTGGACTAATC-3' (antisense). The amplification profile included initial denaturation at 94°C for 5 min, 28 cycles of denaturation at 94°C for 1 min, primer annealing at 62°C for 1 min, extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. The expression of β-actin was used as an internal control for the expression of a constitutively-expressed gene.

One microliter of a 1:100-dilution of the cDNA reaction was amplified using forward and reverse primers for either the EV71 genome or GAPDH as a control, using a Tagman PCR Master Mix (Applied Biosystems, Forster City, CA). The primer and probe sequences used for gene amplification of GAPDH were parts of a commercial Taqman gene expression assay (Applied Biosystems, Cat. No. Hs99999905), while the primers used for the VP1 gene were custom-designed by Applied Biosystems. The amplification profile includes 1 cycle of initial denaturation at 94°C for 5 min, 28 cycles of denaturation at 94°C for 1 min, primer annealing at 62°C for 1 min, extension at 72°C for 1 min, and then 1 cycle of final extension at 72°C for 5 min. The expression of GAPDH was used as an internal control for the assay of a constitutively-expressed gene. The reactions were performed in a StepOnePlus real-time PCR system (Applied Biosystems) under the following thermal cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The threshold-cycle value was normalized to that of GAPDH.

PLASMID OF REPORTER GENES

For construction of the COX-2-luc plasmid, human COX-2 promoter, a region spanning from −483 to +37 bp was cloned into the pGL3-basic vector (Promega). The 5′-flanking region of the human COX-2 gene was cloned using the Genome Walker kit (Clontech, Heidelberg, Germany) with internal (upstream) and external (downstream) primers from the human COX-2 cDNA as follows:

- COX-2 internal primer: 5'-GGTGAGGTGCTCCGAGACCAGCACGG-3' (with a KpnI site).
- COX-2 external primer: 5'-CTCGAGGTGCTCCGAGACCAGCACGG-3' (with an XhoI site).

The point mutation in the NF-κB binding site of the COX-2 promoter was generated by site-directed mutagenesis that splices by overlap extension. To generate CRE mut-luc, two polymerase chain reaction fragments (A and B) were amplified from COX-2-luc, respectively. Fragment A-CRE was generated from a forward primer with a KpnI site (5'-GGTGAGGTGCTCCGAGACCAGCACGG-3') and reverse primer (5'-CAGTCATTTAATCATCAGGGA-3'). Fragment B-CRE was generated from a forward primer (5'-CCCATCAGATTAATGACTG-3') and reverse primer with an XhoI site (5'-CTCGAGGTGCTCCGAGACCAGCACGG-3'). Fragment A was combined with B and this combination was amplified by PCR. The combination of fragments was inserted into pCRII-TOPO (Invitrogen) and was then digested with both KpnI and XhoI. The digested product was inserted into KpnI/XhoI-digested pGL3-basic vector. The resulting plasmid was used to introduce the CRE point mutation (the mutated bases are italicized: TGGTICA→TAATICA).

TRANSFECTION AND PROMOTER ACTIVITY ASSAY

The plasmids were prepared by using QIAGEN plasmid DNA preparation kits (Qiagen, Valencia, CA) and were transfected into SK-N-SH cells using the Lipofectamine reagent (Invitrogen) according to the instructions from the manufacturer. To assess promoter activity, the cells were collected and disrupted by sonication in lysis buffer (25 mM Tris–HCl, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega) according to the instructions provided by the manufacturer. Firefly luciferase activity was standardized for β-galactosidase activity.

SK-N-SH cells were cultured into 12-well plates at 70–80% confluence and were transiently transfected with siRNAs using Lipofectamine. Briefly, siRNA (100 nM) was formulated with Lipofectamine. The transfection complex was diluted into 400 μl of DMEM/F-12 medium and was added directly to the cells. The medium was replaced with DMEM/F-12 containing 2% FBS for 24 h and the cells were then incubated with EV71. Cell lysates were analyzed by Western blotting.

MEASUREMENT OF PGE2 RELEASE

For the detection of PGE2 release, SK-N-SH cells grown to 90% confluence into 12-well culture plates were cultured without serum for 24 h. Growth-arrested cells were incubated with EV71 for the indicated time. When inhibitors were used, they were added 1 h prior
to the application of EV71. After treatment, the supernatants were harvested and stored at −80°C until subsequent assay of PGE2 release. Fifty microliters of supernatant from each sample were used for analysis of PGE2 using a PGE2 EIA kit (Cayman Chemicals) following the manufacturer’s instructions. Briefly, 50 μl of the medium, along with a serial dilution of PGE2 standard samples, were mixed with the appropriate amounts of both the AchE Tracer and the PGE2 antisera, followed by incubation at 4°C on a shaker overnight. After the wells were emptied and rinsed with wash buffer, 200 μl of Ellman’s reagent containing the substrate of acetylcholinesterase was added. The enzyme reaction was carried out on a slow shaker at room temperature for 1.5 h. The absorbance at 405 nm was determined using a Dynatech MR5000 microplate reader (Dynex, Chantilly, VA). Assays were performed in triplicates and normalized to micrograms of proteins.

CO-IMMUNOPRECIPITATION ASSAY

Cell lysates containing 1 mg of proteins were incubated with 2 μg of anti-p300 antibody at 4°C for 1 h, and then 10 μl of 50% protein A-agarose beads was added and mixed for 16 h at 4°C. The immunoprecipitates were collected and washed three times with lysis buffer without Triton X-100; 5X Laemmli buffer was added, and then subjected to electrophoresis on 10% SDS–PAGE. Western blot analysis was performed using an antibody against either p300 or CREB.

CHROMATIN IMMUNOPRECIPITATION ASSAY

SK-N-SH cells in 10-cm dishes were grown to confluence and starved of serum for 24 h after treatment with EV71. Protein-DNA complexes were fixed by 1% formaldehyde in medium. The fixed cells were washed and lysed in SDS-lysis buffer (1% SDS, 5 mM EDTA, 1 mM PMSF, 50 mM Tris–HCl, pH 8.1). The cell lysates were sonicated using a Misonix 3000 sonicator (Misonix, Farmingdale, NY) at 4°C until the DNA size became 200–1,000 base pairs (pulse on for 10 s and off for 12 s at level 4.5). The samples were centrifuged, and the soluble chromatin was pre-cleared by incubation with sheared salmon sperm DNA-protein agarose A slurry (Upstate, NY) at 4°C for 1 h, and then 10 μl of 50% protein A-sepharose (Dynex, Chantilly, VA). Assays were performed in triplicates and analyzed with a one-way ANOVA test to make comparisons with Bonferroni’s test using P < 0.05 as the level of significance. Error bars were omitted when they fell within the dimensions of the symbols.

RESULTS

CREB IS INVOLVED IN EV71-INDUCED COX-2 EXPRESSION

Previous studies have demonstrated that CREB is activated by virus infection, such as SARS-CoV, VSV, and SV [Lallemand et al., 2007; Liu et al., 2007b]. In addition, CREB has been shown to regulate several target genes, including COX-2 [Desdouets et al., 1995; Ahn et al., 1998; Impey et al., 2004; Zhang et al., 2005; White et al., 2006; Klein et al., 2007]. Therefore, we first determined whether EV71-induced COX-2 expression was mediated through CREB signaling in SK-N-SH cells. As shown in Figure 1A, pretreatment with H89 (an inhibitor of PKA which is upstream of CREB) [Chijiwa et al., 1990], attenuated EV71-induced COX-2 expression in a dose-dependent manner. To verify whether CREB was required for COX-2 expression induced by EV71, as shown in Figure 1B, transfection with CREB siRNA down-regulated total CREB protein expression and significantly reduced EV71-induced COX-2 expression. Furthermore, PGE2 production in response to EV71 was markedly reduced in SK-N-SH cells by pretreatment with H89 (Fig. 1C).

Next, we found that the involvement of CREB in COX-2 expression occurred at the transcriptional level since pretreatment with H89 significantly attenuated EV71-induced COX-2 mRNA accumulation (Fig. 1D). To verify the transcriptional regulation of COX-2 gene in this context, SK-N-SH cells were transfected with a luciferase reporter vector containing an exogenous COX-2 promoter, and the cells were then stimulated with EV71 (moi = 5) for 16 h. As shown in Figure 1E, EV71 infection stimulated COX-2 promoter activity which was attenuated by H89. To verify whether the CREB-binding site CRE was required for these responses, the cells were transfected with a mutated COX-2 promoter. As shown in Figure 1F, EV71-stimulated COX-2 promoter activity was decreased by transfection with a mutated CRE (Mt-CRE) in the COX-2 promoter, suggesting that CREB was required for EV71-induced COX-2 expression. Moreover, EV71 stimulated the phosphorylation of CREB in a time-dependent manner (Fig. 1G). In this case, a maximal response was obtained within 30–45 min and then declined to the basal level within 60 min (Fig. 1G). The phosphorylation of CREB was significantly inhibited by pretreatment with H89 (Fig. 1H).

In addition, we compared the effects of EV71 and UV-irradiated EV71 (EV71 was irradiated with 365-nm UV light for 30 min in a 10-cm dish) on these responses. UV-irradiated EV71 could also induce the activation of CREB, indicating that CREB activation required virus binding and entry into the cell, while activation was independent of viral replication (data not shown). These results...
Fig. 1. Regulation of COX-2 expression by EV71 infection occurs through CREB. (A) Cells were incubated with H89 for 1 h, followed by infection with EV71 for 12 h. (B) Cells were transfected with CREB siRNA, and were incubated with EV71 for 12 h. (C) Cells were pretreated with H89 for 1 h before incubation with EV71 for 16 h. (D) Cells were pretreated with H89 for 1 h, followed by incubation with EV71 for 1 h. (E) Cells were transfected with COX-2–luc reporter gene, pretreated with H89 for 1 h, and then incubated with EV71 for 16 h. (F) Cells were transfected with the wildtype COX-2 promoter and CRE mutation COX-2 promoter, prior to incubation with EV71 for 16 h. (G) Cells were incubated with EV71 for the indicated time. (H) Cells were pretreated with H89 for 1 h before stimulation with EV71 for 30 min. The cell lysates were subjected to Western blot analysis using an anti-CREB (B, G, H), anti-COX-2 (A, B), and anti-p-CREB (G, H) antibody. The medium was analyzed for PGE2 release (C). The COX-2 mRNA expression was analyzed by RT-PCR (D). The COX-2 promoter activity was determined in the cell lysates (E, F). Data are expressed as mean ± SEM of at least three independent experiments. *P < 0.05; **P < 0.01 as compared with the cells exposed to EV71 alone (A–E, H). *P < 0.05 as compared with the cells transfected with the wild-type COX-2 promoter in response to EV71 (F). *P < 0.05; **P < 0.01 as compared with the basal level (G).
suggested that the expression of COX-2 and the production of PGE2 induced by EV71 infection required CREB signaling in SK-N-SH cells.

**EV71 INDUCES COX-2 EXPRESSION VIA A c-Src/EGFR/CREB PATHWAY**

Previous studies have reported that CREB activation is mediated through EGFR signaling in various cell types [Amorino et al., 2002; Laag et al., 2006; Ozgen et al., 2008; Pham et al., 2008]. Therefore, we investigated whether EV71-induced CREB phosphorylation was mediated through EGFR signaling. As shown in Figure 2A, pretreatment with an inhibitor of EGFR (AG1478) [Levitzki and Gazit, 1995] attenuated EV71-induced CREB phosphorylation. In addition, as shown in Figure 2B and C, EV71 stimulated a time-dependent phosphorylation of EGFR with a maximal response being observed within 5–10 min, which was inhibited by pretreatment with AG1478. The results indicated that EV71-stimulated phosphorylation of CREB was mediated through EGFR in these cells.

EGFR has been shown to be activated through transactivation by c-Src [Ohtsu et al., 2006]. Thus, we further examined whether EV71-stimulated EGFR phosphorylation was mediated through c-Src in SK-N-SH cells. As shown in Figure 2D, pretreatment with an inhibitor of Src (PP1) [Hanke et al., 1996] attenuated EV71-stimulated EGFR and CREB phosphorylation, revealed by Western blotting. In addition, co-immunoprecipitation experiments were performed to ensure that c-Src directly associated with EGFR, the cells were pretreated with either AG1478 or PP1 for 1 h and followed by incubation with EV71 for 5 min. Co-immunoprecipitation was performed using an anti-c-Src antibody. The results of showed that...
EV71 promoted the association of c-Src and EGFR in a time-dependent manner, which was attenuated by pretreatment with either AG1478 or PP1 (Fig. 2E and F). These results suggested that EV71 activated EGFR mediated through c-Src in SK-N-SH cells. Furthermore, we further verified whether EV71-induced COX-2 expression was also mediated by c-Src/EGFR. As shown in Figure 3A–C, pretreatment with AG1478, EGFR-neutralizing antibody, PP1, or transfection with c-Src siRNA attenuated EV71-induced COX-2 expression, revealed by Western blotting. In addition, PGE₂ production in response to EV71 was markedly reduced in SK-N-SH cells pretreated with either AG1478 or PP1 (Fig. 3D).

We further examined whether the action of c-Src and EGFR on COX-2 expression occurred at the transcriptional level in these cells.

Fig. 3. EV71 infection induces COX-2 expression through c-Src/EGFR in SK-N-SH cells. (A) Cells were pretreated with AG1478 or an EGFR-neutralizing antibody for 1 h, followed by treatment with EV71 for 12 h. (B) Cells were pretreated with PP1 for 1 h, followed by stimulation with EV71 for 12 h. (C) Cells were transfected with c-Src siRNA, and were then incubated with EV71 for 12 h. (D) Cells were pretreated with AG1478 (1 μM) or PP1 (1 μM) for 1 h before incubation with EV71 for 16 h. (E) Cells were pretreated with AG1478 (10 μM) or PP1 (3 μM) for 1 h before incubation with EV71 for 1 h. (F) Cells were transiently transfected with COX-2-luc reporter gene, followed by pretreatment with AG1478 (1 μM) or PP1 (1 μM) for 1 h and incubation with EV71 for 16 h. The cell lysates were subjected to Western blot analysis using an anti-COX-2 (A–C), anti-c-Src (C) antibody. The medium was analyzed for PGE₂ release (D). The RNA samples were analyzed by RTPCR for COX-2 mRNA expression (E). The COX-2 promoter activity was determined in the cell lysates (F). Data are expressed as mean ± SEM of at least three independent experiments. *P < 0.05; #P < 0.01 as compared with the cells exposed to EV71 alone.
As shown in Figure 3E, pretreatment with either AG1478 or PP1 significantly attenuated EV71-induced COX-2 mRNA accumulation. These results indicated that up-regulation of COX-2 mRNA in SK-N-SH cells infected with EV71 was mediated through activation of c-Src and EGFR. This notion was further supported by results indicating that stimulation of COX-2 promoter activity by EV71 was attenuated by pretreatment with either AG1478 or PP1 in SK-N-SH cells transfected with a luciferase reporter vector containing an exogenous COX-2 promoter (Fig. 3F). These results suggested that COX-2 expression and PGE₂ production induced by EV71 infection were mediated through a c-Src/EGFR/CREB cascade in SK-N-SH cells.

EV71 INDUCES CREB PHOSPHORYLATION VIA AN EGFR/P42/P44 MAPK CASCADE

We had previously described that p42/p44 MAPK was involved in the activation of COX-2 expression following EV71 infection in SK-N-SH cells [Tung et al., 2010]. It is known that p42/p44 MAPK is a downstream component of EGFR signaling which can be stimulated by several stimuli in various cell types [Kapoor and O’Rourke, 2003; Ohtsu et al., 2006; Fischer et al., 2007; Rodemann et al., 2007]. Thus, we assessed whether EV71 stimulated p42/p44 MAPK phosphorylation through EGFR. As shown in Figure 4A, EV71-stimulated p42/p44 MAPK phosphorylation was inhibited by pretreatment with AG1478 in a concentration-dependent manner. We further examined whether EV71-induced CREB phosphorylation mediated through p42/p44 MAPK. As shown in Figure 4B, EV71-stimulated CREB phosphorylation was attenuated by pretreatment with an inhibitor of MEK1/2 (U0126) [Favata et al., 1998]. These results suggested that EV71-stimulated CREB phosphorylation was mediated via an EGFR/p42/p44 MAPK pathway.

EV71 INFECTION STIMULATES THE ASSOCIATION OF CREB AND p300 TO THE COX-2 PROMOTER

The p300, a histone acetyltransferase, can interact with CREB to regulate gene expression in various cell types [Chrivia et al., 1993; Kundu et al., 2000; Siu and Jin, 2007]. The association of CREB and p300 leads to enhancement of p300 transcriptional activity and increases the expression of pro-inflammatory genes. Thus, we investigated whether CREB-activated p300 could play a role in EV71-induced COX-2 expression. As shown in Figure 5A, EV71-induced COX-2 expression was significantly attenuated by pretreatment with GR343 (an inhibitor of p300) [Mantelingu et al., 2007]. To further determine whether p300 was required for EV71-induced COX-2 expression, as shown in Figure 5B, transfection with p300 siRNA down-regulated the expression of total p300 protein and significantly reduced COX-2 expression following EV71 infection. Next, co-immunoprecipitation assays were performed to ensure that CREB was directly associated with p300 in response to EV71 (Fig. 5C). To further investigate whether both CREB and p300 were recruited to the promoter region of COX-2 following EV71 infection, ChIP assays were performed using either an anti-CREB or anti-p300 antibody. The cells were incubated with EV71 for the indicated time intervals. Cell lysates were prepared and immunoprecipitated by using either an anti-CREB or anti-p300 antibody. As expected, EV71 stimulated the recruitment of both CREB and p300 to the COX-2 promoter region within 10–30 min, which was attenuated by pretreatment with H89 (Fig. 5D and E). These results suggested that EV71 stimulated the association between CREB and p300 and recruited to COX-2 promoter.

EV71-INDUCED COX-2/PGE₂ EXPRESSION ENHANCES VIRAL REPLICATION THROUGH THE cAMP

We had previously shown that EV71 could auto-regulate its replication by promoting the up-regulation of the COX-2/PGE₂ [Tung et al., 2010]. In addition, the increase of cAMP levels by PGE₂ has been shown to be implicated in the enhancement of viral replication [Dumais et al., 1998; Pyeon et al., 2000; Moriuchi et al., 2001]. Therefore, we examined whether PGE₂ could enhance EV71 replication via cAMP. As illustrated in Figure 6A, treatment of cells with either PGE₂ or forskolin increased the mRNA expression level of the EV71-gene VP1 which was inhibited by pretreatment with H89 (Fig. 6A). These results indicated that generation of cAMP could promote the replication of EV71 in SK-N-SH cells.

It is known that PGE₂ diffuses rapidly outside the cell and can activate specific membrane receptors (EP1–4) which belong to the family of seven-transmembrane-domain G-protein-coupled receptors. Among these receptors, EP2 and EP4 have been shown to interact with the Gs-adenylyl cyclase (AC)-PKA pathway [Yang and Chen, 2008]. Thus, we determined whether EP2 and/or EP4 were involved in PGE₂-mediated EV71 replication. EV71-infected cells were pretreated with 1μM of either AH6809 (EP2 receptor

Fig. 4. EV71 infection induces COX-2 expression through p42/p44 MAPK in SK-N-SH cells. (A) Cells were pretreated with AG1478 for 1 h before stimulation with EV71 for 5 min. (B) Cells were pretreated with U0126 for 1 h, followed by treatment with EV71 for 30 min. The cell lysates were subjected to Western blot analysis using an anti-p44 MAPK, anti-p-p42/p44 MAPK (A), anti-CREB, and anti-p-CREB (B) antibody. Data are expressed as mean ± SEM of at least three independent experiments. *P < 0.05; **P < 0.01 as compared with the cells exposed to EV71 alone.
antagonist) [Keery and Lumley, 1988] or GW627368x (EP4 receptor antagonist) [Wilson et al., 2006] for 1 h and then incubated with 0.3 μM of PGE2. As illustrated in Figure 6B, pretreatment of SK-N-SH cells with either AH6809 or GW627368x inhibited PGE2-induced EV71 replication. These results suggested that PGE2-induced EV71 replication may occur via activation of EP2/EP4 receptors in these cells.

**DISCUSSION**

Although COX-2 expression has been shown to be induced by various viruses [Steer and Corbett, 2003], the intracellular signaling pathways involved in this process was still unknown in EV71-infected SK-N-SH cells. We had previously demonstrated that EV71 induced COX-2 expression and PGE2 production via MAPKs (p42/p44 MAPK, p38 MAPK, and JNK1/2), thereby linking EV71 infection with the activation of NF-κB and AP-1 in SK-N-SH cells [Tung et al., 2010]. However, CREB was also known to play an important role in COX-2 expression in various cell types [Desdouets et al., 1995; Ahn et al., 1998; Lonze et al., 2002; Impey et al., 2004; Zhang et al., 2005; White et al., 2006; Klein et al., 2007]. Previous studies have also reported that viral infection regulates CREB activation [Lallemand et al., 2007; Liu et al., 2007b]. In the present study, our results demonstrated that EV71-induced COX-2 expression was mediated by a cascade implicating c-Src/EGFR/p42/p44 MAPK/CREB in SK-N-SH cells. Based on our findings, we propose a model for the activation of the signaling molecules implicated with COX-2 expression in response to EV71 infection in human neuron cells (Fig. 7).

In the present study, we observed that activation of CREB was essential for EV71-induced COX-2 protein expression, COX-2 mRNA synthesis, and PGE2 production. These processes could be illustrated by the observation that pretreatment of SK-N-SH cells

**Fig. 5.** CREB associates with p300 and the resulting complex binds the COX-2 promoter in SK-N-SH cells infected with EV71. (A) Cells were pretreated with GR343 for 1 h before incubation with EV71 for 12 h. (B) Cells were transfected with p300 siRNA, and were incubated with EV71 for 12 h. (C) Cells were incubated with EV71 for the indicated time. The cell lysates were subjected to immunoprecipitation using an anti-p300 antibody. (D) Cells were incubated with EV71 for the indicated time and ChIP assays were then performed. (E) Cells were pretreated with H89 for 1 h, followed by incubation with EV71 for 30 min. The cell lysates were subjected to Western blot analysis using an anti-COX-2 (A, B), anti-p300 (B) antibody. The immunoprecipitates were then analyzed by Western blot analysis using an anti-p300 or anti-CREB antibody (C). Chromatin was immunoprecipitated with anti-CREB or anti-p300 antibody and 1% of the precipitated chromatin was assayed to verify equal loading ("Input") (D, F). Data are expressed as mean ± SEM of at least three independent experiments. *P < 0.05; **P < 0.01 as compared with the cells exposed to EV71 alone (A, B, E). *P < 0.05; **P < 0.01 as compared with the basal level (C, D).
with H89 attenuated EV71-induced COX-2 expression and PGE2 production. In addition, transfection with CREB siRNA also significantly reduced EV71-induced COX-2 expression (Fig. 1A–D). We also used a CRE-(MtCRE)-mutated COX-2 promoter and demonstrated that mutants of CRE-binding site significantly blocked EV71-induced COX-2 promoter activity (Fig. 1F). We also demonstrated that EV71 infection stimulated CREB activation, while this effect was diminished by pretreatment with H89, suggesting that CREB was involved in EV71-induced COX-2 expression in SK-N-SH cells (Fig. 1G and H). These results are consistent with previous studies which demonstrated that protein S from SARS-CoV regulates COX-2 expression through the CREB pathway in HEK293T, COS-7, or A549 cells [Liu et al., 2007b].

EGFR signaling has been shown to regulate CREB-dependent gene expression [Amorino et al., 2002; Laag et al., 2006; Ozgen et al., 2008; Pham et al., 2008]. In the present study, the activation of CREB by EV71 infection was blocked by pretreatment with AG1478, suggesting that EV71 induced CREB activation in an EGFR-dependent manner [Fig. 2A]. Previous studies have reported that PP1 blocks EGFR transactivation stimulated by glucagon-like peptide 1 in pancreatic β-cells [Ohtsu et al., 2006]. Our results indicated that c-Src was involved in EV71-induced EGFR and CREB activation since pretreatment with PP1 attenuated the phosphorylation of these signaling proteins. In addition, we demonstrated that EV71 activated the association of c-Src and EGFR, which was blocked by pretreatment with either AG1478 or PP1 (Fig. 2E and F). Therefore, transactivation of EGFR could represent one of the basic mechanisms underlying the mitogenic activity of EV71 in SK-N-SH cells. Moreover, COX-2 protein expression, PGE2 production, COX-2 mRNA synthesis, and COX-2 promoter activity were induced by EV71 infection, which were inhibited by pretreatment with AG1478, an EGFR-neutralizing antibody, PP1, or transfection with c-Src siRNA [Fig. 3A–F]. These results suggest that EV71-induced COX-2 expression is mediated through a c-Src/EGFR/CREB signaling pathway in SK-N-SH cells.

Several studies have reported that Akt and p42/p44 MAPK were activated following stimulation of EGFR by different stimuli in various cell types [Kapoor and O'Rourke, 2003; Ohtsu et al., 2006; Fischer et al., 2007; Rodemann et al., 2007]. In the present study, pretreatment of SK-N-SH cells with AG1478 inhibited EV71-stimulated p42/p44 phosphorylation, indicating that activation of EGFR was required for this cellular response [Fig. 4A]. However, the phosphorylation of CREB induced by EV71 infection was blocked by
pretreatment with U0126 (Fig. 4B), but not by treatment with other MAPK inhibitors, such as SB203580 (inhibitor of p38 MAPK) and SP600125 (inhibitor of JNK1/2) (data not shown). These results were consistent with previous studies which demonstrated that the total cell number of both Rat-2 fibroblast cells and BrdU-positive cells increased following treatment with lysophosphatidic acid (LPA) via a p42/p44 MAPK/CREB pathway [Kwon et al., 2009]. We suggested that activation of the p42/p44 MAPK signaling pathway was required for CREB activation. Nonetheless, we also demonstrated that EV71 induced the activation of Akt by using inhibitors of PI-3K (LY294002) or AG1478. In addition, pretreatment with LY294002 or transfection with Akt siRNA significantly attenuated the activation of Akt and COX-2 expression following EV71 infection in SK-N-SH cells. However, pretreatment with LY294002 had no significant effect on EV71-stimulated CREB phosphorylation (data not shown). Taken together, these results indicated that EV71-induced COX-2 expression was dependent on CREB mediated through c-Src/EGFR/p42/p44 MAPK, but not c-Src/EGFR/Akt in SK-N-SH cells.

It has been demonstrated that p300 interacts with CREB to regulate gene expression [Chrivia et al., 1993; Kundu et al., 2000; Siu and Jin, 2007]. In this study, pretreatment with GR343 attenuated EV71-induced COX-2 expression in SK-N-SH cells. In addition, transfection with p300 siRNA also significantly reduced EV71-induced COX-2 expression, suggesting that p300 was essential for the production of the COX-2 protein following EV71 infection (Fig. 5A and B). It was found that the CREB complex containing p300 was recruited to the COX-2 promoter region in response to EV71, which was inhibited by pretreatment with H89 (Fig. 5D and E). These results were consistent with previous studies indicating that phorbol 12-myristate 13-acetate (PMA), interleukin-1β (IL-1β), or lipopolysaccharide (LPS) was able to induce the association between p300 and CREB, which was involved in the regulation of COX-2 promoter activity [Deng et al., 2004].

Previously, we have shown that the expression of COX-2 and the production of PGE2 following EV71 infection could enhance viral replication [Tung et al., 2010]. However, the modulatory effects of COX-2 and PGs on viral replication in EV71-infected cells remained unclear. In this study, we demonstrated that EV71 replication in SK-N-SH cells was increased by treatment with PGE2, or forskolin, which was significantly inhibited by treatment with H89 (Fig. 6A). Our data indicated that PGE2 might be essential for the replication of EV71 through cAMP signaling in SK-N-SH cells, consistent with previous studies which report that increase of cAMP by PGE2 promotes viral replication [Dumais et al., 1998; Pyeon et al., 2000; Moriuchi et al., 2001]. Moreover, we established that the effect of PGE2 on EV71 replication was mediated through both EP2 and EP4 receptors, since pretreatment with either AH6809 or GW627368x inhibited PGE2-induced viral replication (Fig. 6B). In this case, it is possible that the formation of cAMP following EV71 infection was lower than following direct PGE2 stimulation, and that the level of EV71-induced cAMP was insufficient for viral replication.

On the basis of observations published in the literature and the findings presented here, we propose a signaling model to explain the downstream events that occur following infection of SK-N-SH cells by EV71. As depicted in Figure 7, EV71 stimulates the activation of EGFR through c-Src and leads to phosphorylation of p42/p44 MAPK. The MAPK p42/p44 in turn promotes the activation of CREB. Furthermore, CREB associates with p300 and the resulting complex is recruited to the COX-2 promoter region to activate COX-2 promoter activity, mRNA accumulation, and protein expression. In summary, our findings suggested that EV71-induced COX-2 expression may be mediated through c-Src/EGFR/p42/p44 MAPK/CREB signaling in SK-N-SH cells. Based on our earlier findings [Tung et al., 2010], EV71 was shown to induce the expression of COX-2 and the production of PGE2 in SK-N-SH cells through the concerted action of c-Src, EGFR, MAPKs, NF-κB, AP-1, and CREB. The production of PGE2 following EV71 infection further promoted viral replication through EP2/EP4 receptors-cAMP signaling. Therefore, pharmacological approaches which target COX-2 and its upstream signaling components may provide useful strategies to limit the deleterious effects of CNS inflammation during viral infection.

ACKNOWLEDGMENTS

This work was supported by the grants CMRPD170333, CMRPD150313, CMRPD140253, CMRPD170492, and CMRPD180371 from Chang Gung Medical Research Foundation and by the grants NSC96-2320-B-182-003, NSC96-2320-B-182-047-MY3, and NSC97-2321-B-182-007 from National Science Council, Taiwan.

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