BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 23(6), 539-548 (2015)

EP2 Induces p38 Phosphorylation via the Activation of Src in HEK 293 Cells

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Abstract

Prostaglandin E_2 (PGE_2), a major product of cyclooxygenase, binds to four different prostaglandin E_2 receptors (EP1, EP2, EP3, and EP4) which are G-protein coupled transmembrane receptors (GPCRs). Although GPCRs including EP receptors have been shown to be associated with their specific G proteins, recent evidences suggest that GPCRs can regulate MAPK signaling via non-G protein coupled pathways including Src. EP2 is differentially expressed in various tissues and the expression of EP2 is induced by extracellular stimuli. We hypothesized that an increased level of EP2 expression may affect MAPK signaling. The overexpression of EP2 in HEK 293 cells resulted in significant increase in intracellular cAMP levels response to treatment with butaprost, a specific EP2 agonist, while overexpression of EP2 alone did not increase intracellular cAMP levels. However, EP2 overexpression in the absence of PGE_2 induced an increase in the level of p38 phosphorylation as well as the kinase activity of p38, suggesting that up-regulation of EP2 may promote p38 activation via non-G protein coupled pathway. Inhibition of Src completely blocked EP2-induced p38 phosphorylation and overexpression of Src increased the level of p38 phosphorylation, indicating that Src is upstream kinase for EP2-induced p38 phosphorylation. EP2 overexpression also increased the Src activity and EP2 protein was co-immunoprecipitated with Src. Furthermore, sequential co-immunoprecipitation studies showed that EP2, Src, and β-arrestin can form a complex. Our study found a novel pathway in which EP2 is associated with Src, regulating p38 pathway.

Key Words: EP2, Prostagladin, p38, beta-arrestin, GPCR

INTRODUCTION

Prostanoids including prostaglandins (PGs) and thromboxanes (TXs) are arachidonic acid metabolites that are synthesized by the sequential actions of cyclooxygenase (COX) and specific PG synthases. Prostanoids regulate local cellular homeostasis through binding to their specific receptors. Among prostanoids, PGE_2 is the most abundantly produced in many type of tissues and is associated with many physiological functions or pathological conditions. PGE_2 receptors (EPs) are G-protein coupled receptor (GPCR) and four different EP receptors (EP1, 2, 3, and 4) have been identified. EP receptors are differentially expressed in various tissues and coupled with various G proteins and effector molecules, regulating their downstream signaling pathways. For example, EP1 receptor is coupled to Gαq and induces an increase in cytosolic calcium concentration. The EP3 receptor signaling is associated with Gαi and decreases intracellular cAMP levels. EP2 and EP4 receptors are coupled to Gαs, resulting in an increase in intracellular cAMP levels (Regan, 2003).

Early studies have shown that the stimulation of a GPCR with its agonist results in exchange of GDP for GTP in Gα subunit, leading to dissociation of Gα subunit from Gβ and Gγ subunit and subsequent activation of various effector molecules for second messenger generation such as cAMP. However, recent studies showed that some GPCRs activate MAPK pathways independent of heterotrimeric G protein activation (Sun *et al*., 2007b). For example, Src has been shown to be involved in GPCR-mediated Erk activation (Cao *et al*., 2000; Sun *et al*., 2007a). While some GPCRs provide direct binding sites for Src (Cao *et al*., 2000; Sun *et al*., 2007a), others recruit adaptor molecules such as β-arrestins for binding and activation of Src (DeFea *et al*., 2000). In addition, EP4 has been shown to form a complex with β-arrestin 1 and Src in response to PGE_2, resulting in an activation of EGFR (Buchanan *et al*., 2006). However, the association of EP2 with Src or β-arrestin

Open Access **http://dx.doi.org/10.4062/biomolther.2015.043**

Received Apr 22, 2015 **Revised** Jul 14, 2015 **Accepted** Aug 17, 2015
Published online Nov 1, 2015

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has not been studied well and the role of EP2 receptor in p38 signaling is not clear.

The level of EP2 expression is induced by extracellular stimuli or in pathologic conditions and the increased level of EP2 may affect downstream signaling pathway. LPS treatment highly induces the expression of EP2 and EP4 receptors (Hubbard *et al*., 2001; Ikegami *et al*., 2001) in macrophages and this induction was suggested to be involved in regulation of TNF-α (Katsuyama *et al*., 1998). EP2 expression is highly induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor promoter, and is increased in papilloma and carcinoma by at least 15-fold over vehicle-treated skin (Sung *et al*., 2006). Overexpression of EP2 receptor in epidermis of EP2 transgenic mice resulted in enhanced skin tumor formation, proliferation, and blood vessel formation (Sung *et al*., 2006).

In this report, we hypothesized that the increased level of EP2 expression may affects MAPK signaling. We found that EP2 overexpression without PGE_2 induces an increase in phosphorylation and kinase activity of p38 while increases in intracellular cAMP levels are dependent on agonist stimulation. We further showed that EP2 overexpression induces phosphorylation of p38 via Src and that EP2 forms a complex with Src and β-arrestin. Our results provide evidence for an additional level of EP2 signaling in which EP2, Src, and β-arrestin regulates p38 signaling pathway.

MATERIALS AND METHODS

Materials

Endoglycosidase H, PNGase F, and λ-phosphatase were purchased from NEB (Ipswich, MA, USA). Butaprost (free acid) was purchased from Cayman Chemical (Ann Arbor, MI, USA). SB203580, H89, Ly294002, PP2, SQ22536, and AG1478 were purchased from Calbiochem (Billerica, MA, USA). NSC23766 was purchased from Tocris bioscience (Bristol, UK).

Plasmids

Human EP1-4, human Hsp27, and mouse Src were amplified from commercially available cDNA constructs with Phusion high fidelity DNA polymerase (NEB) and subcloned into pFLAG-NEH, pHA-NEH, and pMyc-NEH, all of which are a pcDNA3.1 (-) with FLAG, HA, or Myc tag at its 5' end of cloning site so that expressed protein has a tag at its N-terminal end. Human β-arrestin 1 and β-arrestin 2 were amplified and subcloned into pMyc-CEH which is a pcDNA3.1 (-) containing a Myc tag at its 3' end of cloning site. All plasmids were verified with sequencing. pcDNA3-HA-rErk2 was kind gift from Dr. Melanie Cobb (University of Texas Southwestern Medical Center) and pCMV-FLAG-p38 was kind gift from Dr. Roger Davis (University of Massachusetts Medical School).

Cell culture and transfection

HEK293 human embryonic kidney cells were purchased from American Type Culture Collection and cultured in DMEM media supplemented with 10% fetal bovine serum (Hyclone) and 10 μg/ml gentamycin (Invitrogen, Waltham, MA, USA). One day before transfection, 1×10^6 cells were plated in each well of a 6 well cell culture plate. Cells were transfected with total 3-4 μg of plasmid per well using Lipofectamine 2000 (Invitrogen).

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the TRIZOL reagent (Life technologies, Gaithersburg, MD). Single-strand cDNA was synthesized from 2 μg of total RNA using SuperScript II reverse transcriptase (Life technologies, Gaithersburg, MD). The cDNAs for EP1-4 and actin were amplified by PCR with specific primers. The sequences of the forward and reverse primers were as follows: EP1 (forward) 5'-ctgcccatcttctccatgac-3' and (reverse) 5'- cgggtactgcagctcatag-3'; EP2 (forward) 5'-ttctcaacctcatccgcatg-3' and (reverse) 5'-cctaaggatggcaaagaccc-3'; EP3 (forward) 5'-tatgcgagccacatgaagac-3' and (reverse) 5'-cataagctgaatggccgtct-3'; EP4 (forward) 5'-cttactcattgccacctccc-3' and (reverse) 5'-cctccaaggccattttcact-3'; actin (forward) 5'-ccttctacaatgagctgcgt-3' and (reverse) 5'-ctccttaatgtcacgcacga-3', respectively. RT-PCR was performed using the following cycling conditions: 95°C for 3 min followed by 17 cycles (actin) and 28 cycles (EP receptors) of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec with a final extension at 72°C for 5 min. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide.

Western blot analysis

Forty-eight hours after transfection, cells were harvested and lysed in RIPA buffer. Lysates were cleared by centrifugation (12,000 rpm, 4°C, 10 min) and protein concentration was measured using BCA protein assay kit (Pierce, Waltham, MA, USA). Equal amounts of protein were solubilized and heated at 65°C in LDS sample buffer (Invitrogen) with sample reducing agent (Invitrogen) for 10 min, and then separated by SDS-PAGE. The separated proteins were transferred to an Immobilon-P membrane (EMD Millipore, Billerica, MA, USA). Following incubation in blocking buffer (TBS with 5% nonfat dry milk and 0.1% Tween 20) for one hour at room temperature, the membranes were probed overnight at 4°C. The membranes were washed and then probed with a horseradish peroxidase-linked secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for one hour at room temperature. Detection was made with an enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA) followed by exposure of membrane to film. All transfection and western blot analysis was conducted at least three times. Antibodies that were used are phosphor (p)-p38 (Cell Signaling Technology), p-Hsp27 (Cell Signaling Technology), p-Erk (Cell Signaling Technology), p-JNK (Cell Signaling Technology), p-Src Family (Cell Signaling Technology), Src (Cell Signaling Technology), p-EGFR (Cell Signaling Technology), EGFR (Cell Signaling Technology), anti-FLAG® M2-HRP (Sigma-Aldrich, St Louis, MO, USA), anti-c-Myc-HRP (GenScript, Piscataway, NJ, USA), and anti-HA-HRP (Roche Life Sciences, Indianpolis, USA).

Measurement of intracellular cAMP

One day before transfection, 1×10^6 cells were plated in each well of a 6 well cell culture plate. Cells were transfected with total 3-4 μg of plasmid per well using Lipofectamine 2000 (Invitrogen). Forty-two hours after transfection, cells were stimulated with 1 μM butaprost (Cayman chemical) for six hours and harvested in 0.1 M HCl. cAMP concentration was measured with cAMP EIA kit (Cayman chemical) according to manufacturer's protocol.

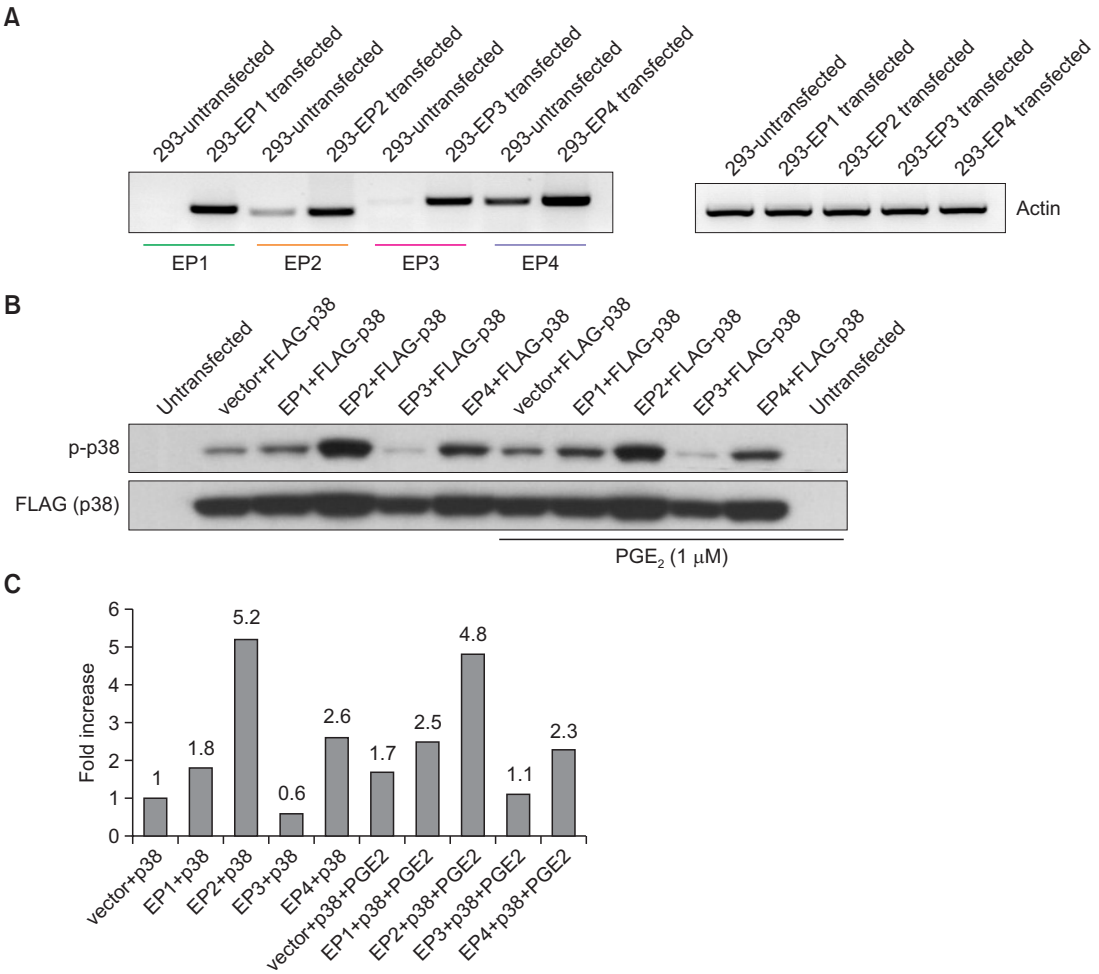
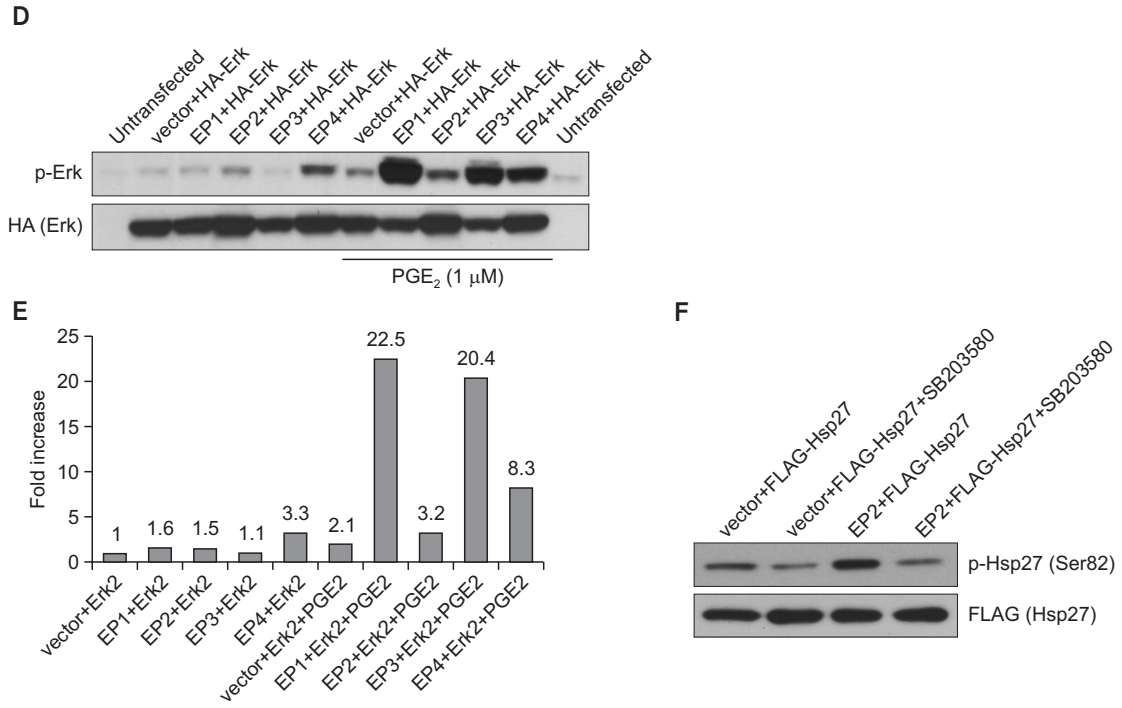


Fig. 1. Overexpression of EP2 protein induces phosphorylation of p38 in a PGE_2 independent-manner. (A) HEK 293 cells were transfected with empty vector and pcDNA3.1 (-) EP1, 2, 3, or 4. Forty-eight hours after transfection, total RNA was isolated and RT-PCR analysis was performed using specific primers for each EP receptor. (B) EP2 overexpression induces phosphorylation of p38 in a PGE_2-independent manner. HEK 293 cells were transfected with empty vector, pcDNA3.1 (-) EP1, 2, 3, or 4 and pCMV-FLAG-p38. Forty-eight hours after transfection, cells were treated with or without 1 μM PGE_2 for 30 min and cell lysates were subjected to western blot analysis for p-p38. Membranes were stripped and reprobed for FLAG to confirm equal expression of p38. (C) Graphical representation of Fig. 1B. The result from Fig. 1B was subjected to semi-quantitative analysis using Scion image software. The ratio of phosphorylated/total p38 was obtained and the results were normalized to untreated vector transfected cells. (D) EP2 overexpression does not significantly affect Erk phosphorylation. HEK 293 cells were transfected with empty vector, pcDNA3.1 (-) EP1, 2, 3, or 4 and pcDNA3-HA-rErk2. Forty-eight hours after transfection, cells were treated with or without 1 μM PGE_2 for 30 min and cell lysates were subjected to western blot analysis for p-Erk. Cell lysates from untransfected HEK 293 cell were included. Membranes were stripped and reprobed for HA to confirm equal expression of Erk. (E) Graphical representation of Fig. D. The ratio of phosphorylated/total Erk2 was obtained and the results were normalized to untreated vector transfected cells. (F) EP2 overexpression increases endogenous p38 activity. HEK 293 cells were transfected with empty vector or pcDNA3.1 (-) EP2 and pcDNA3.1 (-)-FLAG-Hsp27. Twenty-four hours after transfection, cells were incubated with or without 20 μM of SB203580 for another 24 hours. Cell lysates were subjected to western blot analysis for p-Hsp27. Membranes were stripped and reprobed for FLAG to confirm equal expression of Hsp27.

Co-immunoprecipitation

One day before transfection, 4.5×10^6 cells were plated in P100 cell culture dishes. Cells were transfected with total 20-24 μg of plasmid per dish using Lipofectamine 2000. Forty-eight hours after transfection, cells were washed with PBS and overlayed with 1.5 ml of co-immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5 % NP-40). Cells were incubated for 30 min at 4°C and then scraped into 2 ml microcentrifuge tubes. Lysates were cleared by centrifugation (12,000 rpm, 4°C, 5 min) and protein concentration was measured using BCA protein assay kit (Pierce). Equal amounts of lysates were added to 15 ml conical tubes which contained 50 μl of pre-washed anti-FLAG® M2-agarose (Sigma-Aldrich) or anti-Myc tag antibody (Cell Signaling Technology) and anti-Mouse IgG-Agarose (Sigma-Aldrich). After mixing at 4°C for 2 hours, agarose beads were washed 6 times with 10 ml of co-immunoprecipitation buffer and bound proteins were eluted with 20 μl of 2x LDS sample buffer.



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Sequential co-immunoprecipitation

One day before transfection, 4.5×10^6 cells were plated in P100 cell culture dishes. Cells were transfected with total 20-24 μg of plasmid per dish using Lipofectamine 2000. Forty-eight hours after transfection, cells were washed with PBS and overlayed with 1.5 ml of co-immunoprecipitation buffer. Cells were incubated for 30 min at 4°C and then scraped into 2 ml microcentrifuge tubes. Lysates were cleared by centrifugation (12,000 rpm, 4°C, 5 min) and protein concentration was measured using BCA protein assay kit (Pierce). Equal amounts of lysates were added to 15 ml conical tubes containing 50 μl of pre-washed anti-FLAG® M2-agarose and rotated for 1 hour at 4°C. Agarose beads were washed 4 times with 10 ml of co-immunoprecipitation buffer and incubated with 1 ml of co-immunoprecipitation buffer with 120 μg/ml FLAG peptide (Sigma-Aldrich) for 30 min at 4°C. Tubes were centrifuged (1,200 rpm for 5 min at 4°C) and supernatants were collected in 15 ml tubes which contained 3 μl of anti-Myc tag antibody and pre-washed anti-Mouse IgG-Agarose. Tubes were rotated for 1 hour at 4°C and agarose beads were washed 6 times with 10 ml of co-immunoprecipitation buffer. Bound proteins were eluted with 20 μl of 2x LDS sample buffer.

RESULTS

EP2 overexpression increases the phosphorylation of p38 in HEK 293 cells in a PGE_2-independent manner

GPCRs are known to affect cell growth by activating multiple mitogen activated protein kinase (MAPK) cascades (O'Hayre *et al*., 2014). Because PGE_2 is involved in MAPK signaling, including Erk and p38 (Bos *et al*., 2004; Faour *et al*., 2006; Zhang *et al*., 2007), we examined the role of each EP receptor on p38 and Erk signaling. To determine the role of each EP receptor on p38 signaling, HEK 293 cells were co-transfected with expression vector for each EP receptor and a FLAG-tagged p38 expression vector and then treated with PGE_2. Expression of each EP receptor was confirmed by RT-PCR analysis (Fig. 1A). HEK 293 cells expressed endogenous EP2 and EP4 mRNA while the expression of EP1 and EP3 mRNA was not detectable (Fig. 1A). As shown in Fig. 1B and C, the overexpression of EP2 resulted in significant increase in p38 phosphorylation compared to that in empty vector-transfected cells, while EP1 and EP4 slightly increased p38 phosphorylation. However, PGE_2 treatment did not induce further increase in phosphorylation of p38 in EP2-transfected cells, suggesting that overexpression of EP2 may promote p38 activation in a G-protein independent manner.

In order to provide the evidence that EP2 overexpression specifically induces p38 phosphorylation, we measured the effect of EP2 overexpression on Erk phosphorylation. HEK 293 cells were co-transfected with expression vectors for individual EP receptors and a HA-tagged Erk2 expression vector and then treated with PGE_2. In contrast to p38, overexpression of EP2 in the absence of PGE_2 marginally increased the Erk phosphorylation, while EP4 overexpression alone significantly induced Erk phosphorylation (Fig. 1D and E). The most significant increases in PGE_2-induced Erk phosphorylation were observed in EP1- and EP3-transfected cells, while PGE_2 treatment slightly increased Erk phosphorylation in EP2-transfected cells, suggesting that EP2 overexpression specifically promotes the phosphorylation of p38. Then we focused on the EP2-p38 pathway. Because phosphorylation of p38 leads to activation of p38, we examined whether EP2-induced p38 phosphorylation is associated with an increase in p38 kinase activity.

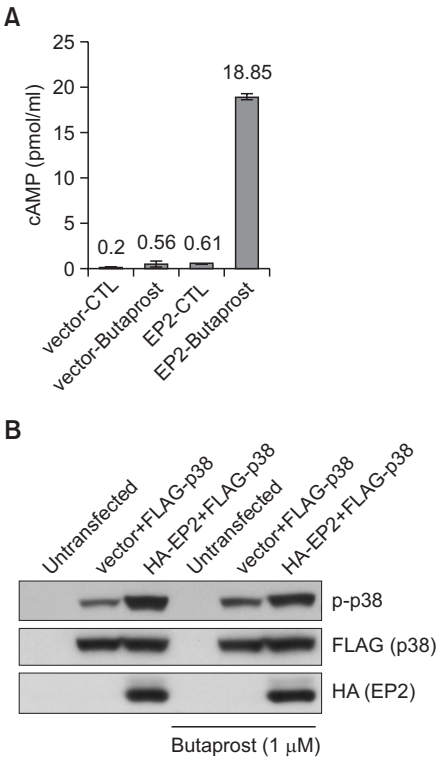

Fig. 2. Overexpression of EP2-induced p38 phosphorylation does not depend on the activation of G protein. (A) EP2 agonist treatment significantly increases intracellular cAMP concentration in EP2 expressing cells. HEK 293 cells were transfected with empty vector or EP2 expression plasmid and forty-two hours after transfection, cells were incubated with or without butaprost (1 μM) for 6 hours. Intracellular cAMP level was measured from cell lysates. (B) Butaprost treatment does not affect EP2-induced p38 phosphorylation. HEK 293 cells were transfected with empty vector or pcDNA3.1 (-) HA-EP2 and pCMV-FLAG-p38. Forty-eight hours after transfection, cells were treated with or without 1 μM butaprost for 30 min and cell lysates were subjected to western blot analysis for p-p38. Membranes were stripped and reprobed for FLAG and HA to confirm equal expression of p38 and EP2.

Studies have shown that Hsp27 is a substrate of the p38 kinase and that the activation of p38 results in Hsp27 phosphorylation at Ser82, leading to changes in its intracellular distribution and interaction with other proteins (Rouse *et al*., 1994; Chang and Karin, 2001; Williams, 2001; Yuan and Rozengurt, 2008). As shown in Fig. 1F, incubation of Hsp27-transfected cells with SB203580, a widely used p38-specific inhibitor, reduced the basal level of Hsp27 phosphorylation at Ser82 (lane 2) compared to that in untreated cells (lane 1). Moreover, co-transfection of HEK 293 cells with EP2 and Hsp27 increased Hsp27 phosphorylation (lane 3) while SB203580 abolished EP2-induced Hsp27 phosphorylation at Ser82 (lane 4). This result suggests that overexpression of EP2 is associated with the increased p38 activity.

Overexpression of EP2-induced p38 phosphorylation is not dependent on the activation of heterotrimeric G protein

EP2 receptor is coupled to Gαs which increases intracellular cAMP levels upon agonist stimulation (Regan, 2003; Castellone *et al*., 2005). We next examined if overexpression of EP2 induces an increase in intracellular cAMP levels response to agonist stimulation. In order to prevent activation of other EP receptors, we stimulated vector or EP2-transfected HEK 293 cells with butaprost, a specific EP2 agonist. As shown in Fig. 2A, a slight increase in intracellular cAMP level was observed in vector transfected cells after butaprost treatment as well as untreated EP2-transfected cells. In contrast, when EP2-transfected HEK 293 cells were treated with butaprost, a 95 fold increase in intracellular cAMP level over untreated vector samples was observed (Fig. 2A), indicating that the expressed EP2 protein binds to its agonist and activates Gαs protein.

We then determined if EP2-induced p38 phosphorylation is further increased by butaprost treatment. However, as seen in PGE_2-treated cells (Fig. 1B and C), butaprost treatment did not further increase EP2-induced p38 phosphorylation (Fig. 2B), suggesting that EP2 induced phosphorylation of p38 is independent of the heterotrimeric G protein activity.

EP2 overexpression induces phosphorylation of p38 in a Src-dependent manner

Some GPCRs activate the MAPK pathway independent of G protein by interacting with Src. In addition, EP receptors are associated with the activation of PI3K, PKC, and PKA and phosphorylation of p38 is induced by AMP-activated protein kinase (AMPK) (Xi *et al*., 2001) and Rac (Uddin *et al*., 2000). We suspected that the increased level of EP2 expression may activate one of these signaling pathways, resulting in an increase in p38 phosphorylation. To determine the upstream kinase involved in EP2-induced p38 phosphorylation, HEK 293 cells were co-transfected with EP2 and p38 in the presence of different kinase inhibitors. As shown in Fig. 3A, PP2, a specific Src inhibitor, treatment completely blocked the EP2-induced p38 phosphorylation while the other inhibitors were not effective, suggesting that Src may be the potential upstream kinase involved in overexpression of EP2-induced p38 phosphorylation. Because inhibition of Src blocked EP2-induced p38 phosphorylation, we anticipated that overexpression of Src would induce phosphorylation of p38. As shown in Fig. 3B, Src overexpression significantly increased phosphorylation of p38 and the Src-induced phosphorylation of p38 is completely blocked by PP2 pre-treatment. These results indicate that the EP2-induced p38 phosphorylation may be mediated by Src.

EP2 activates and interacts with Src

Src is known to be expressed in HEK293 cells (Abrahamsen *et al*., 2003; Arinsburg *et al*., 2006). Consistent with these studies, we found the expression of Src in HEK293 cells (Fig. 4B). The phosphorylation of Tyr416 in the activation loop of Src protein is associated with the increased activity (Hunter, 1987). When HEK 293 cells were transfected with EP2, the levels of endogenous p-Src were not significantly altered due to the low transfection efficiency with transient transfection (data not shown). Therefore, we co-transfected HEK293 cells with EP2 and Src expression plasmids and the effect of EP2 on Src phosphorylation was examined. Because the previous results suggest that Src is a potential upstream kinase for EP2-induced p38 phosphorylation, we anticipated that overexpression of EP2 would increase Src activity. When HEK 293 cells were co-transfected with expression vectors for EP2 and Src, p-Src (Tyr416) levels were increased compared to those in vector and Src-transfected cells (Fig. 4B), indicating that

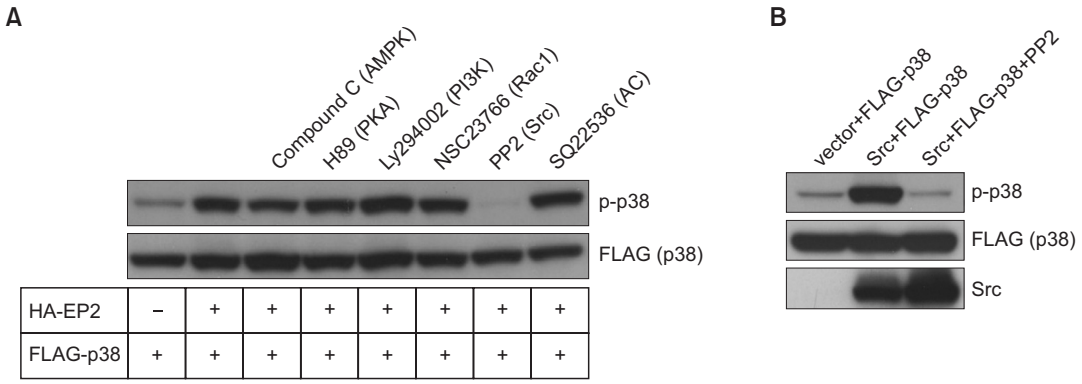

Fig. 3. Overexpression of EP2-induced p38 phosphorylation is mediated by Src. (A) Src inhibitor blocks EP2-induced increase in p38 phosphorylation. HEK 293 cells were transfected with empty vector or pcDNA3.1 (-) EP2 and pCMV-FLAG-p38. Twenty-four hours after transfection, cells were incubated in the presence of different inhibitors for another 24 hours and cell lysates were subjected to western blot analysis for phospho-p38. Compound C (20 μM):AMP-activated protein kinase inhibitor, H89 (10 μM):protein kinase A inhibitor, Ly294002 (20 μM):PI3K inhibitor, NSC23766 (100 μM):Rac1 inhibitor, PP2 (10 μM):Src inhibitor, SQ22536 (200 μM):adenylate cyclase inhibitor. Membranes were stripped and reprobed for FLAG to confirm equal expression of p38. (B) Src expression increases phosphorylation of p38. HEK 293 cells were transfected with empty vector or pcDNA3-Src and pCMV-FLAG-p38. Twenty-four hours after transfection, cells were incubated with or without PP2 (10 μM) for another 24 hours and cell lysates were subjected to western blot analysis for p-p38. Membranes were stripped and reprobed for FLAG followed by Src to confirm equal expression of p38 and Src.

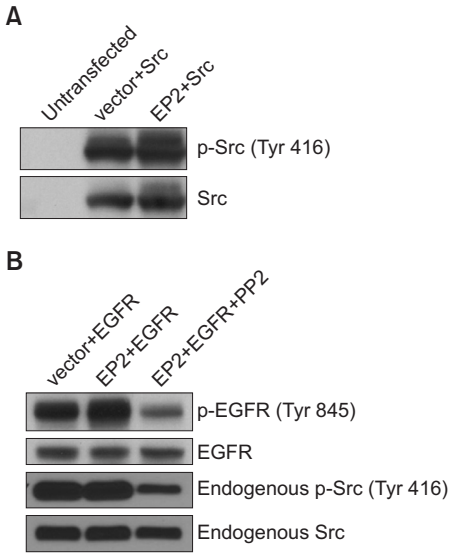

Fig. 4. EP2 overexpression increases Src phosphorylation and activity. (A) EP2 overexpression increases Src phosphorylation. HEK 293 cells were transfected with empty vector or pcDNA3.1(-) EP2 and pcDNA3-Src. Cell lysates were subjected to western blot analysis for p-Src (Tyr416). Membranes were stripped and reprobed for Src to confirm equal expression of Src. (B) EP2 overexpression increases endogenous Src activity. HEK 293 cells were transfected with empty vector or pcDNA3.1(-) EP2 and pcDNA3-EGFR. Twenty-four hours after transfection, cells were incubated with or without PP2 for another 24 hours. Cell lysates were subjected to western blot analysis for p-EGFR (Tyr845). Membranes were stripped and reprobed for EGFR to confirm equal expression of EGFR. Additionally, cell lysates were subjected to western blot analysis for p-Src and total Src to examine the effect of PP2 on endogenous Src phosphorylation in HEK 293 cells.

EP2 overexpression induces the phosphorylation of Src. Then we examined if EP2 overexpression increases Src activity. Because Tyr845 in EGFR is a substrate for Src and known to be phosphorylated by Src (Biscardi *et al*., 1999; Tice *et al*., 1999), we hypothesized that EP2 would activate Src, which in turn results in increase in EGFR phosphorylation at Tyr845. To test this, HEK293 cells were co-transfected with expression vectors for EP2 and EGFR and incubated with or without PP2. Western blot analysis for phosphorylated Tyr845 in EGFR (Fig. 4B) showed that the phosphorylation was increased by EP2 overexpression compared to vector and EGFR-transfected cells. Furthermore, EP2-induced increase in Tyr845 phosphorylation was blocked by PP2 treatment, indicating that EP2 overexpression increases endogenous Src activity. These results demonstrate that EP2 induces the phosphorylation and the activation of Src.

EP2 forms a complex with Src and β-arrestin

Since EP2 overexpression increased Src activity, we determined if EP2 interacts with Src. HEK293 cells were co-transfected with FLAG-tagged EP2 and Myc-tagged Src and co-immunoprecipitation study was performed. As shown in Fig. 5A, EP2 and Src were co-immunoprecipitated, indicating that EP2 binds to Src. In addition, β-arrestin has been shown to recruit Src to some GPCRs (Luttrell *et al*., 1999) and the association of EP4-β-arrestin1-Src complex has been shown to induce the activation of EGFR and Akt (Buchanan *et al*., 2006). Based on this information, we tested the possibility that β-arrestin 1 or 2 may be the part of EP2-Src complex. We showed that EP2 co-immunoprecipitates with Src (Fig. 5A). Next, we examined if β-arrestin cooperates with EP2 for p38 phosphorylation. As shown in Fig. 5B, overexpression of β-arrestin 1 or 2 further increased EP2-induced p38 phosphorylation (lane 5 and 6), while overexpression of β-arrestin 1 or 2 alone did not affect the level of p38 phosphorylation (lane 3 and 4), suggesting that β-arrestin may interact with EP2.

Previously we showed that EP2 co-immunoprecipitates with Src (Fig. 5A). We also found that β-arrestin forms a complex with EP2 or Src (data not shown). However, these results do not provide enough evidence for the existence of EP2/β-arrestin/Src complex. In order to determine if EP2 forms a

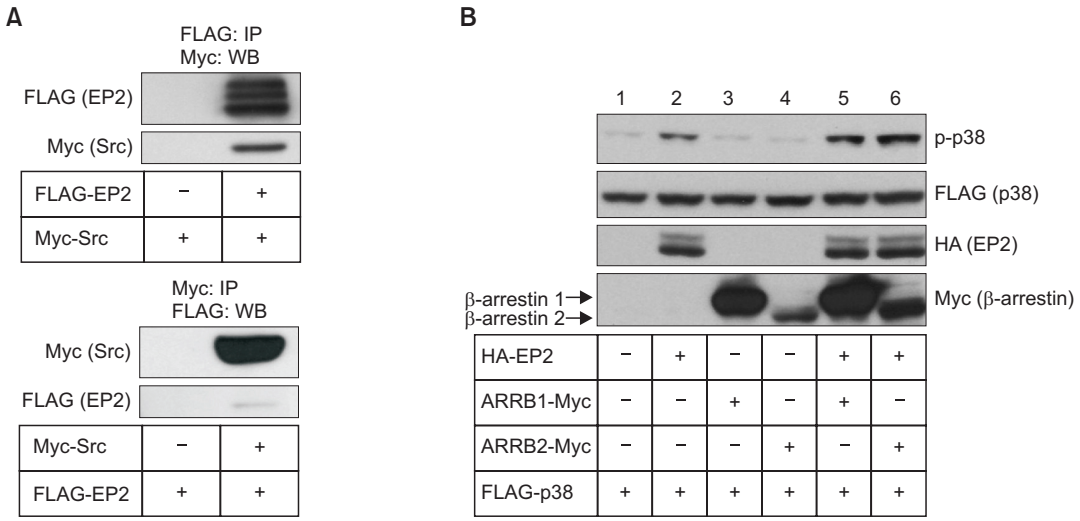

Fig. 5. β-Arrestin further increases overexpression of EP2-induced p38 phosphorylation and forms a complex with EP2 and Src. (A) EP2 and Src interact with each other. (Left) HEK 293 cells were transfected empty vector or pcDNA3.1(-) FLAG-EP2 and pcDNA3.1 (-) Myc-Src. Forty-eight hours after transfection, cell lysates were immunopreciptated for FLAG with FLAG antibody-agarose. Immunoprecipitated proteins were subjected to western blot analysis for Myc. (Right) HEK 293 cells were transfected empty vector or pcDNA3.1(-) Myc-Src and pcDNA3.1 (-) FLAG-EP2. Forty-eight hours after transfection, cells lysates were immunopreciptated for Myc with Myc antibody. Immunoprecipitated proteins were subjected to western blot analysis for FLAG. (B) The overexpression of β-arrestin 1 or 2 further increases overexpression of EP2-induced p38 phosphorylation. HEK 293 cells were co-transfected with (i) empty vector and pCMV-FLAG-p38, (ii) pcDNA3.1 (-) HA-EP2 and pCMV-FLAG-p38, (iii) pcDNA3.1 (-) ARRB1-Myc and pCMV-FLAG-p38, (iv) pcDNA3.1 (-) ARRB2-Myc and pCMV-FLAG-p38, (v) pcDNA3.1 (-) HA-EP2, pcDNA3.1 (-) ARRB1-Myc, and pCMV-FLAG-p38, and (vi) pcDNA3.1 (-) HA-EP2, pcDNA3.1 (-) ARRB2-Myc, and pCMV-FLAG-p38. Forty-eight hours later, cell lysates were subjected to western blot analysis for p-p38. Membranes were stripped and sequentially reprobed for FLAG, HA, and Myc to confirm equal expression of p38, EP2, and β-arrestins. (C) Experimental scheme of sequential co-immunoprecipitation. (D) Src forms a complex with EP2 and β-arrestin. HEK 293 cells were co-transfected with (i) pcDNA3.1 (-) FLAG-EP2 and pcDNA3.1 (-) HA-Src, (ii) pcDNA3.1 (-) ARRB1-Myc and pcDNA3.1 (-) HA-Src, (iii) pcDNA3.1 (-) FLAG-EP2, pcDNA3.1 (-) ARRB1-Myc, and pcDNA3.1 (-) HA-Src, (iv) pcDNA3.1 (-) ARRB2-Myc and pcDNA3.1 (-) HA-Src, and (v) pcDNA3.1 (-) FLAG-EP2, pcDNA3.1 (-) ARRB2-Myc, and pcDNA3.1 (-) HA-Src. Forty-eight hours after transfection, cell lysates were immunopreciptated for FLAG using FLAG antibody-agarose. Immunoprecipitated proteins were then eluted under non-denaturing condition using FLAG peptide. FLAG peptide-eluted proteins were immunoprecipitated with Myc antibody. Myc-immunopreciptated proteins were subjected to western blot analysis for HA, FLAG, and Myc (right panel). The lysates used for sequential co-immunoprecipitation were included to confirm equal expression of EP2, Src, and β-arrestins (left panel). The location of β-arrestin 1 and 2 were shown (arrows).

complex with β-arrestin and Src, we conducted sequential co-immunoprecipitation (Fig. 5C). HEK 293 cells were transfected with FLAG-tagged EP2, Myc-tagged β-arrestin 1 or 2, and HA-tagged Src expression vectors and lysates were first immunoprecipitated with FLAG antibody-agarose to immunoprecipitate FLAG-EP2/HA-Src, FLAG-EP2/β-arrestin-Myc, and FLAG-EP2/HA-Src/β-arrestin-Myc complexes, leaving β-arrestin-Myc/HA-Src complex in solution. FLAG-immunoprecipitated proteins were then eluted under the non-denaturing condition with FLAG peptide. FLAG peptide-eluted proteins were then immunoprecipitated with Myc antibody. This second immunoprecipitation will bring down FLAG-EP2/β-arrestin-Myc and FLAG-EP2/β-arrestin-Myc/HA-Src complexes and eliminate FLAG-EP2/HA-Src complex. Finally, Myc-immunoprecipitated proteins were subjected to western blot analysis for HA to determine if HA-Src co-immunoprecipitates with FLAG-EP2 and β-arrestin-Myc.

Seuqential co-immunoprecipitation only detects EP2/β-arrestin/Src complex and not EP2/β-arrestin, EP2/Src, or β-arrestin/Src complexes. HA-Src was included in all transfection to prove that co-imunoprecipitation is specific and the level of HA-Src expression was not different among transfected cells (Fig. 5D, left panel). The result from sequential co-immunoprecipitation shows that Src was co-immunoprecipitated with EP2 and β-arrestin 1 or 2 (lane 3 and 5), indicating that EP2, Src, and β-arrestin form a complex.

DISCUSSION

While PGE_2 mediates diverse physiological or pathological functions, the actual cellular response to PGE_2 often depends on the cell type. This suggests that the level of each EP receptor may determine the cellular effects of PGE_2. Previous studies have shown that the EP expression is induced by extracellular stimuli or in pathological conditions. LPS treatment highly induced the expression of EP2 and EP4 receptors (Hubbard *et al*., 2001; Ikegami *et al*., 2001) in macrophages and EP2 mRNA was significantly induced by TPA in mouse skin (Sung *et al*., 2006). In addition, the level of EP4 expression was increased in colon cancers compared to surrounding normal tissues (Chell *et al*., 2006). These reports suggest that increased level of certain EP receptor may be associated with changes in cellular responses to PGE_2 or an increased EP expression may affect cellular signaling.

Studies with overexpression of GPCRs have been conducted to examine the mechanism of receptor activation and to determine functional consequence of receptor expression

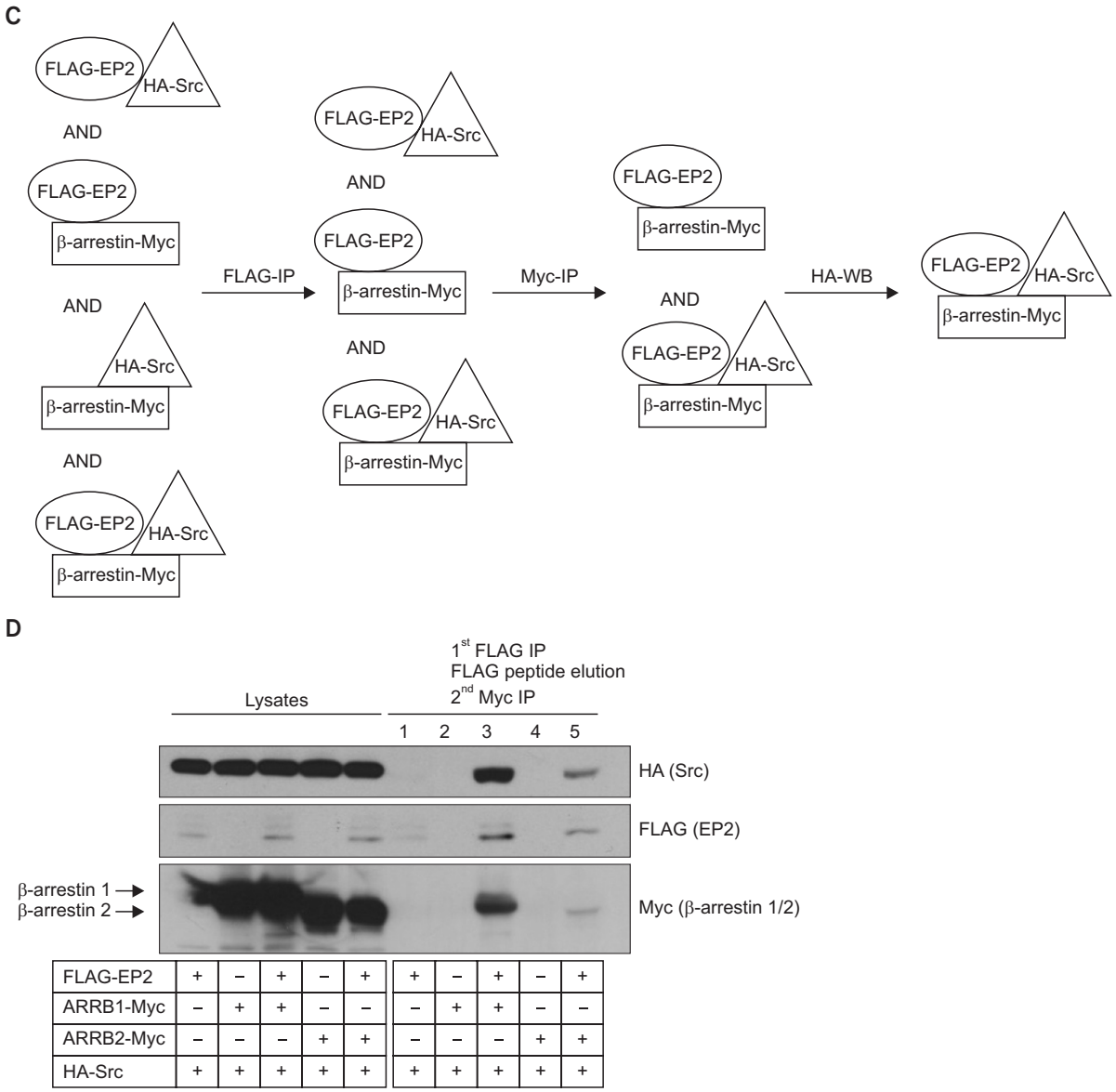

Fig. 5. Continued.

including EP2 and EP4 (Fujino *et al*., 2002; Fujino *et al*., 2003). We wanted to determine the role of each EP receptor on MAPK signaling by overexpression of each EP receptor and examine the response to PGE_2 in the transfected cells. In our experiments, overexpression of EP2 significantly induced phosphorylation of p38, while it had marginal effect on Erk phosphorylation. However, when stimulated with PGE_2 or butaprost, we were not able to observe further increase in p38 phosphorylation, while butaprost treatment significantly increased intracellular cAMP level compared to untreated vector-transfected cells (Fig. 2A). These results suggest that the effect of EP2 on p38 phosphorylation may not depend on activation of heterotrimeric G proteins. Consistent with these results, co-transfection with Gαs, Gβ, Gγ, or Gβ and Gγ had no or marginal effect on p38 phosphorylation (data not shown). Furthermore, inhibition of adenylate cyclase or PKA had no effect on EP2-induced p38 phosphorylation (Fig. 3A). Based on these results, we concluded that EP2-induced p38 phosphorylation is independent of G protein activation. In addition, although all EP receptors are associated with Erk activation, EP1 and EP3 had most dramatic effect on Erk phosphorylation in response to PGE_2 stimulation. Further studies are ongoing to determine signaling pathways which is involved in EP1 or EP3-induced Erk activation and functional significance.

We found that EP2 overexpression induced an increase in phosphorylation and activity of p38 and this finding suggests possibility that EP2 overexpression may modulate inflammatory response by regulating p38 activity. p38 plays an important role in inflammation by modulating the expression of pro-inflammatory mediators such as enzymes, cytokines, or metalloproteases (MMPs) via transcriptional or post-transcriptional mechanism (Kumar *et al*., 2003). EP2 transgenic mouse

skin showed increased inflammation and angiogenesis (Sung *et al*., 2006) and the combined inhibition of EP2 and EP4 resulted in suppression of inflammation in a collagen-induced arthritis model (Honda *et al*., 2006). These reports support our hypothesis in which increased EP2 signaling may amplify inflammatory responses by activating p38 kinase. In addition, we observed that EP2 overexpression induced an increase in the level of IL-8 and MMP-13 mRNA in a p38-dependent manner (data not shown). Future studies will be performed to examine the role of EP2 and p38 in modulation of inflammatory processes.

We have demonstrated that Src is involved in EP2-induced p38 phosphorylation (Fig. 3A and B). Consistent with our finding, Src has been suggested to be involved in p38 activation during EGF-induced VEGF production in pancreatic cancer cells (Summy *et al*., 2005) and in lysophosphatidic acid-mediated p38 activation (Estrella *et al*., 2007). However, the mechanism by which Src induces phosphorylation of p38 remains to be determined. We examined if EP2-induced p38 phosphorylation involves EGFR pathway because Src interacts with EGFR. However, treatment with AG1478, a potent EGFR kinase inhibitor, did not affect EP2-induced p38 phosphorylation and overexpression of EGFR marginally affected p38 phosphorylation compared to Src (data not shown), suggesting that EP2-induced p38 phosphorylation does not involve EGFR pathway. Phosphorylation of p38 is subject to kinase cascades and MKK3 or MKK6 are the main MKKs that activate p38 (Derijard *et al*., 1995; Raingeaud *et al*., 1996). However, other possible mechanisms have been suggested for activation of p38 such as an interaction of p38 with TAB-1 (Transforming growth factor-Activated kinase 1 Binding protein 1) (Ge *et al*., 2002) and conformational change due to phosphorylation at Tyr323 (Salvador *et al*., 2005). Whether Src directly phosphorylates p38 or activates other intermediate kinase for p38 is not clear.

Overexpression of EP2 increased phosphorylation and activity of Src (Fig. 5A) and EP2 formed a complex with Src. Some GPCRs such as AngII type 1 receptor (AT1R) (Yin *et al*., 2003) and β-Adrenergic receptors (β2-AR) (Ma and Huang, 2002) can activate Src. Among the EP receptors, EP4 has been shown to recruit Src through β-arrestin and result in EGFR activation (Buchanan *et al*., 2006). β-Arrestin is important in GPCR signaling and binds to activated GPCR, preventing further interaction of GPCR with its effector molecules (Hanyaloglu and Zastrow, 2008) or linking GPCR to other signaling molecules (Lefkowitz and Whalen, 2004; Shenoy and Lefkowitz, 2005). We have shown that β-arrestin cooperated with EP2 for p38 phosphorylation and the result from sequential co-immunoprecipitation experiment indicates that β-arrestin may be the part of EP2-Src complex, although we were not able to show if endogenous EP2 can bind to Src and β-arrestin because of the lack of reliable EP2 antibody.

In summary, our results indicate that the increased level of EP2 allows the binding with Src and β-arrestin and induces p38 phosphorylation/activation. Our studies provide the insights on additional level of EP2 signaling and a possible link between EP2 signaling and inflammation via p38. In addition, our finding suggests that the regulation of EP2 expression is important in controlling the p38 inflammatory pathway independent of PGE_2 levels. Thus, agents or drugs that downregulate the expression of EP2 could have anti-inflammatory responses and EP2 could be a new target for the development of anti-inflammatory drugs without the adverse side effects of selective COX-2 inhibitors.

ACKNOWLEDGMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (2014R1A2A2A01004698) and the National Institutes of Health [grant number 5R00ES016757-04].

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