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RESEARCH PAPER

NF-κB-dependent IL-8 induction by prostaglandin E_2 receptors EP_1 and EP_4

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BACKGROUND AND PURPOSE

Recent studies suggested a role for PGE_2 in the expression of the chemokine IL-8. PGE_2 signals via four different GPCRs, EP_1-EP_4. The role of EP_1 and EP_4 receptors for IL-8 induction was studied in HEK293 cells, overexpressing EP_1 (HEK-EP_1), EP_4 (HEK-EP_4) or both receptors (HEK-EP_1 + EP_4).

EXPERIMENTAL APPROACH

IL-8 mRNA and protein induction and IL-8 promoter and NF-κB activation were assessed in EP expressing HEK cells.

KEY RESULTS

In HEK-EP_1 and HEK-EP_1 + EP_4 but not HEK or HEK-EP_4 cells, PGE_2 activated the IL-8 promoter and induced IL-8 mRNA and protein synthesis. Stimulation of HEK-EP_1 + EP_4 cells with an EP_1-specific agonist activated IL-8 promoter and induced IL-8 mRNA and protein, whereas a specific EP_4 agonist neither activated the IL-8 promoter nor induced IL-8 mRNA and protein synthesis. Simultaneous stimulation of HEK- EP_1 + EP_4 cells with both agonists activated IL-8 promoter and induced IL-8 mRNA to the same extent as PGE_2. In HEK-EP_1 + EP_4 cells, PGE_2-mediated IL-8 promoter activation and IL-8 mRNA induction were blunted by inhibition of IκB kinase. PGE_2 activated NF-κB in HEK-EP_1, HEK-EP_4 and HEK-EP_1 + EP_4 cells. In HEK-EP_1 + EP_4 cells, simultaneous activation of both receptors was needed for maximal PGE_2-induced NF-κB activation. PGE_2-stimulated NF-κB activation by EP_1 was blocked by inhibitors of PLC, calcium-signalling and Src-kinase, whereas that induced by EP_4 was only blunted by Src-kinase inhibition.

CONCLUSIONS AND IMPLICATIONS

These findings suggest that PGE_2-mediated NF-κB activation by simultaneous stimulation of EP_1 and EP_4 receptors induces maximal IL-8 promoter activation and IL-8 mRNA and protein induction.

Abbreviations

AP-1, activator protein 1; C/EBP, CCAAT/enhancer-binding protein; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CXCR, CXC motif chemokine receptor; $EP_{1\text{-}4}$, PGE_2 receptor subtype 1–4; G protein, guanine nucleotide-binding protein; GAPDH, glycerinaldehyde 3-phosphate dehydrogenase; IKK, IκB kinase; $InsP_3$, inositol trisphosphate; MIP, macrophage inflammatory protein; PI3K, phosphoinositide 3 kinase; Wort, wortmannin

Introduction

Interleukin-8 (IL-8, CXCL8) is a 72 amino acid pro-inflammatory CXC chemokine, which is expressed in many different cell types, including endothelial and epithelial cells as well as inflammatory cells such as monocytes, macrophages and T-cells. One of its major physiological functions is the recruitment of neutrophils from the blood to the tissue

after infection or injury. Through its function as potent chemoattractant peptide, IL-8 plays a major role in the initiation and maintenance of inflammatory responses (Harada *et al.*, 1994). In addition, IL-8 was shown to be involved in angiogenesis and tumour progression (Waugh and Wilson, 2008). In adipocytes IL-8 attenuated insulin-stimulated Akt-phosphorylation by a MAPK-dependent pathway (Kobashi *et al.*, 2009). IL-8 mediates its biological effects by binding to two different GPCRs CXCR1 and CXCR2. Both receptors signal predominantly through Gαi, and pertussis toxin attenuates most IL-8 functions (Hall *et al.*, 1999).

IL-8 expression is regulated predominantly on the transcriptional level. The transcription rate is controlled by a short promoter region reaching from –11 to –133 within the 5′-flanking region of the IL-8 gene (Hoffmann *et al.*, 2002). Binding sites for the transcription factors NF-κB, activator protein 1 (AP-1) and CCAAT/enhancer-binding protein (C/EBP) are located in this short promoter. Whereas AP-1 and C/EBP activation are not always needed for IL-8 induction, NF-κB activation is required for IL-8 expression in nearly all cell types studied (Hoffmann *et al.*, 2002). IL-8 expression varies over a considerable range within the same cell type. In some cell types, IL-8 expression is induced more than 100-fold in response to pro-inflammatory stimuli such as TNFα and IL-1 (Roebuck, 1999; Hoffmann *et al.*, 2002). At the site of inflammation, both resident and infiltrating cells produce prostaglandins in addition to these cytokines (Rajakariar *et al.*, 2006). Experiments with non-selective COX inhibitors and COX-2-selective inhibitors have suggested that prostanoids have a role in the regulation of IL-8 synthesis. Thus, the non-specific COX-1/COX-2 inhibitor aspirin suppressed TNFα-stimulated IL-8 expression in human umbilical vein endothelial cells (Yang *et al.*, 2004). Similarly, the COX-2 inhibitor NS398 suppressed IL-8 formation in *Helicobacter pylori*-treated gastric cancer cells and inhibited IL-8 expression in bradykinin-stimulated airway epithelial cells. (Rodgers *et al.*, 2002; Takehara *et al.*, 2005). PGE_2, which is the most abundant prostaglandin at sites of inflammation, acts via binding to four specific G protein-coupled PGE_2 receptors called PGE_2 receptor subtype 1–4 (EP_1-EP_4). EP_1 is coupled to an as yet unknown G protein. Binding of PGE_2 to EP_1 leads to a transient increase in intracellular calcium concentrations (Katoh *et al.*, 1995) as well as to activation of PLC presumably by coupling to a Gq protein (Ji *et al.*, 2010). EP_2 and EP_4 are coupled to Gs, and activation of theses receptors leads to an increase in cAMP and activation of PKA (Breyer *et al.*, 2001). In addition, activation of EP_4 receptors but not EP_2, can stimulate phosphoinositide 3 kinase (PI3K), which subsequently leads to phosphorylation and activation of Akt kinase (Fujino *et al.*, 2003). The G protein coupling of EP_3 is more promiscuous. This receptor has different C-terminal splice variants that signal via a decrease in cAMP (Gi-coupling) and/or an increase in inositol trisphosphate ($InsP_3$) and Ca^{2+} (Gq-coupling).

The aim of the current study was therefore, firstly, to elucidate if PGE_2 is able to activate NF-κB via distinct EP receptors leading to IL-8 expression and, secondly, to analyse the signal transduction pathways linking G protein-coupled EP receptors to NF-κB activation. To this end, EP_1 and EP_4, which were shown in previous studies to be involved in PGE_2-stimulated IL-8 formation (Caristi *et al.*, 2005; Dey and Chadee, 2008; Vij *et al.*, 2008; Dey *et al.*, 2009), were overexpressed in HEK293 cells alone or in combination. Then PGE_2 stimulated activation of the IL-8 promoter as well as IL-8 mRNA and protein induction were determined, and activation of NF-κB in these cells was analysed. It was found that the activation of both EP_1- and EP_4-dependent signal chains by PGE_2 was needed to elicit maximal activation of the transcription factor NF-κB, maximal IL-8 promoter activation as well as IL-8 mRNA and protein induction.

Methods

Materials

All chemicals were purchased from commercial sources indicated throughout the text. Oligonucleotides were custom-synthesized by Eurofins MWG/Operon (Ebersberg, Germany).

EP receptor specific agonists ONO-D1-004 (EP_1 agonist) and ONO-AE1-329 (EP_4 agonist) (Suzawa *et al.*, 2000) were kindly provided by ONO Pharmaceutical Co, Ltd, Osaka, Japan. Antibodies used were phospho-IKKα (Ser^{180})/IKKβ (Ser^{181}), IκB kinase (IKK) α, IKKβ, phospho-Src (Tyr^{416}) and Src from cell signalling (Frankfurt, Germany).

Cell culture and treatment

HEK293 cells were cultured in DMEM containing 10% FCS and antibiotics. HEK293 cells stably expressing human EP_1 or EP_4 were established as described previously (Neuschäfer-Rube *et al.*, 2004) and maintained in HEK293 culture medium supplemented with 0.5 mg mL^{-1} G-418 as selection marker. Double transgenic cells expressing human EP_1 and EP_4 were obtained by transfecting HEK-EP_1 cells with a pcDNA3.1-Zeo-hEP4 expression construct. Double transgenic cell clones were selected by the addition of 0.1 mg mL^{-1} Zeocin (CAYLA, Toulouse, France) as a second selection marker.

Cell surface ligand binding

Cells in 24-well plates (1×10^5 cells per well) were washed once with a HEPES buffered salt solution (15 mM HEPES, 4,7 mM KCl, 1,2 mM KH_2PO_4, 1 mM glucose, 2.2 mM $CaCl_2$) and incubated for 2 h at 4°C with 100 µL of 5 nM [^{3}H]-PGE_2 ± 10 µM PGE_2 to determine non-specific binding in the same buffer. To determine the contribution of EP_1 or EP_4 receptors to total [^{3}H]-PGE_2 binding in HEK-EP_1 + EP_4 cells, 1 µM of specific agonists were added in addition to labelled PGE_2. Plates were washed three times with ice-cold HEPES buffered salt solution and cell-associated radioactivity was released by lysing cells in 400 µL 0.3 M NaOH containing 1% (w v^{-1}) SDS. The radioactivity in the cell lysates was counted in 5 mL Rotiszint solution (Roth, Karlsruhe, Germany).

Real-time reverse transcription (RT)-PCR

Cells were stimulated with 1 µM PGE_2, 1 µM of EP receptor agonists or 50 ng mL^{-1} TNFα for the time indicated and washed with PBS. Total RNA was isolated from treated cells using GeneMatrix Universal RNA kit (EURx, Gdansk, Poland). The 1–5 µg total RNA was reverse transcribed into cDNA using a mixture of oligo dT and random nucleotide primers and a M-MuLV reverse transcriptase (Fermentas, St. Leon Rot, Germany). Hot start real-time PCR for the quantification of

Table 1
Oligonucleotide primers for qPCR

Gene	Forward	Reverse
GAPDH	5′-TGATGACATCAAGAAGGTGG	5′-TTACTCCTTGGAGGCCATGT
IL-8	5′-CAGTTTTGCCAAGGAGTGCTAA	5′-AACTTCTCCACAACCCTCTGC
EP_1	5′-TCGCTTCGGCCTCCACCTTCTTTG	5′-CGTTGGGCCTCTGGTTGTGCTTAG
EP_2	5′-CGAGACGCGACAGTGGCTTCC	5′-CGAGACGCGGCGCTGGTAGA
EP_3	5′-CGGGGCTACGGAGGGGATGC	5′-ATGGCGCTGGCGATGAACAACGAG
EP_4	5′-TCGCGCAAGGAGCAGAAGGAGACG	5′-GGACGGTGGCGAGAATGAGGAAGG

Accession numbers for the genes were: GAPDH (AB062273), IL-8 (AK311874), EP_1 (L22647), EP_2 (NM_000956), EP_3 (E15918) and EP_4 (NM_000958).

each transcript was carried out using 2× Maxima SybrGreen qPCR mix (Fermentas), 0.25 μM of each primer and 2.5 μL–5 μL of cDNA that was diluted 1:10. PCR was performed with an initial enzyme activation step at 95°C for 10 min, followed by 42 cycles of denaturation at 95°C for 30 s, annealing at 57°C (GAPDH and IL-8) or 63°C (EP receptor) for 30 s and extension at 72°C for 1 min in a real-time DNA thermal cycler (iCycler™, 20 μL reaction volume or CFX96™, 10 μL reaction volume, BIO-RAD, Munich, Germany). The oligonucleotides used are listed in Table 1. The expression level was calculated as *n*-fold induction of the gene of interest (int) in treated versus control cells with GAPDH (gap) as a reference gene. The calculation is based on the differences in the threshold cycles between control (*c*) and treated (*t*) groups according to the formula: fold induction = $2^{(c-t)\text{int}}/2^{(c-t)\text{gap}}$. For the calculation of EP receptor copy numbers, plasmids with cloned cDNAs coding for EP receptor and GAPDH were used as a template to prepare standard curves with defined copy numbers.

IL-8 ELISA

Cells were stimulated with 1 μM PGE_2, 1 μM EP receptor agonists or 50 ng ml^{-1} TNFα for the time indicated. After the incubation, supernatants were collected and processed for IL-8 quantification by sandwich ELISA as previously described (Hippenstiel *et al.*, 2000).

Cell transfection and luciferase reporter gene assay

Cells were transfected with pGL3-basic based luciferase reporter gene plasmids NF-κB-Luc (Clontech, Madison, WI, USA) or IL-8prom-Luc (Nourbakhsh *et al.*, 2001). HEK293 cells, and HEK293 cells stably expressing EP_1, EP_4 or both receptors were transfected using a modified calcium phosphate transfection protocol. Twenty hours after transfection, cells were treated with 1 μM PGE_2, 1 μM EP receptor agonists or 50 ng mL^{-1} TNFα for the time indicated. At the end of the experiment, cells were lysed in 100 μL lysis buffer, and firefly luciferase activity was measured in 25 μL of cell lysate using the Fluostar Optima (BMG Labtech, Offenburg, Germany).

Western blot analysis

HEK293-EP_1 + EP_4 cells were stimulated with 1 μM PGE_2 or EP receptor specific agonist for the time indicated. In some experiments, cells were incubated with 10 μM of the Src kinase inhibitor (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl) pyrazolo[3,4-d]pyrimidine (PP2) for 1 h before agonist stimulation. Cells were lysed in lysis buffer [20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v v^{-1}) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 50 mM NaF, protease inhibitors and 1 mM sodium orthovanadate], homogenized by sonication, and insoluble material was removed by centrifugation (10 000× *g*, 15 min, 4°C). Protein content was determined. Proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked in 5% non-fat dry milk in 20 mM Tris, 136 mM NaCl and 0.1% (v v^{-1}) Tween (TBS/Tween) for 1 h at room temperature and incubated with the first antibody in TBS/Tween containing 5% BSA overnight at 4°C and a horseradish-peroxidase-conjugated anti-rabbit IgG for 2 h at room temperature. Visualization of immune complexes was performed by using chemoluminescence reagent.

Statistical analysis

Unless otherwise indicated, data were analysed by Student's two sided *t*-test for unpaired samples in either homoscedastic or heteroscedastic mode, as appropriate. The threshold for significance was set at $P < 0.05$.

Results

Characterization of EP_1 or/and EP_4 expressing HEK293 cells

HEK, HEK- EP_1, HEK- EP_4 or HEK- EP_1 + EP_4 cells were cultured for 24 h and then EP receptor mRNA copy numbers were determined by real-time RT-PCR using defined copy numbers of EP receptor/GAPDH containing plasmids for the preparation of standard curves. Untransfected HEK cells expressed all four EP receptor mRNAs at a very low level in the order: $EP_1 < EP_3 < EP_2 = EP_4$ (Table 2). The absolute copy numbers indicate that untransfected HEK cells express no functional EP_1 or EP_3, but they might express low amounts of functional EP_2 and EP_4. In HEK- EP_1 and HEK- EP_4 cells, the respective EP receptor mRNAs were highly overexpressed. Overexpression of either EP_1 or EP_4 left EP_2 and EP_3 mRNA levels unaffected. The EP_4 mRNA copy number in HEK- EP_4 cells was twice the

Table 2

EP receptor mRNA profiles in HEK, HEK-EP_1, HEK-EP_4 and HEK-EP_1 + EP_4 cells

Cells	EP_1 (EP_1 mRNA × 1000/GAPDH mRNA)	EP_2 (EP_2 mRNA × 1000/GAPDH mRNA)	EP_3 (EP_3 mRNA × 1000/GAPDH mRNA)	EP_4 (EP_4 mRNA × 1000/GAPDH mRNA)
HEK	0.01 ± 0	0.23 ± 0.14	0.03 ± 0.01	0.20 ± 0.11
HEK-EP1	169 ± 51	0.25 ± 0.1	0.03 ± 0.01	0.19 ± 0.05
HEK-EP4	0.02 ± 0.01	0.2 ± 0.11	0.01 ± 0	313 ± 91
HEK-EP1 + EP4	117 ± 55	0.25 ± 0.13	0.01 ± 0	291 ± 135

HEK293 cells stably expressing EP_1, EP_4 or EP_1 + EP_4 were cultured for 24 h. EP receptor mRNA and GAPDH mRNA were measured by real-time RT-qPCR as described in Methods. Plasmids (10^2–10^8 copies) containing EP receptor or GAPDH cDNAs were used for preparing standard curves for the calculation of EP receptor or GAPDH mRNA copy numbers. Data represent the mean ± SEM of at least three independent RNA preparations. EP receptor mRNA contents are expressed as copy number EP receptor mRNA × 1000/copy number GAPDH mRNA.

Table 3

Competition of cell surface [^{3}H]-PGE_2-binding by PGE_2 and receptor-specific agonist in HEK, HEK-EP_1, HEK-EP_4 and HEK-EP_1 + EP_4 cells

Cells	Competition of [^{3}H]-PGE_2 binding by PGE_2 (fmol per 10^5 cells)	Competition of [^{3}H]-PGE_2 binding by EP_1 agonist (fmol per 10^5 cells)	Competition of [^{3}H]-PGE_2 binding by EP_4 agonist (fmol per 10^5 cells)
HEK	0.05 ± 0.09	–	–
HEK-EP_1	3.99 ± 0.71	–	–
HEK-EP_4	15.76 ± 0.63	–	–
HEK-EP_1 + EP_4	6.15 ± 0.41	0.37 ± 0.10	5.81 ± 0.39

HEK, HEK-EP_1, HEK-EP_4 or HEK-EP_1 + EP_4 cells in 24-well plates (1×10^5 cells per well) were incubated with 5 nM [^{3}H]-PGE_2 ± 1 μM of EP receptor-specific agonists for 2 h at 4°C. Non-specific cell surface binding was determined in the presence of 10 μM PGE_2. Unbound ligand was removed, and cell surface bound [^{3}H]-PGE_2 was measured by lysing the cells and counting the radioactivity in the lysate. The [^{3}H]-PGE_2 binding by specific EP receptors was determined by subtracting [^{3}H]-PGE_2 binding in the presence of agonists from specific [^{3}H]-PGE_2 binding. Data represent the mean ± SEM of three independent experiments.

EP_1 mRNA copy number in HEK- EP_1 cells. In HEK-EP1 + EP4 cells mRNAs of each individual receptor was similar to the copy number in mono-transgenic cells (Table 2). To verify that up-regulation of EP1/EP4 mRNAs resulted in overexpression of functional receptor proteins, cell surface [^{3}H]-PGE_2 binding was measured. In accordance with the low expression level of EP receptor mRNAs, untransfected HEK cells showed only little specific [^{3}H]-PGE_2 binding. (Table 3). By contrast, HEK- EP_1 cells bound about 4 fmol [^{3}H]-PGE_2 per 10^5 cells (80-fold more than untransfected HEK cells) and HEK-EP_4 about 16 fmol [^{3}H]-PGE_2 per 10^5 cells (315-fold more than untransfected HEK cells). HEK- EP_1 + EP_4 cells bound about 6 fmol [^{3}H]-PGE_2 per 10^5 cells; thus, the total number of specific PGE_2 binding sites was 123-fold higher than in untransfected HEK cells. The increase in mRNA levels following double transfection was hence not translated into a similar increase in total PGE_2-binding sites. In addition, competition binding experiments with EP_1 or EP_4 specific agonists in HEK-EP_1 + EP_4 cells revealed that most of the specific [^{3}H]-PGE_2 binding sites consisted of EP_4 (94%) and EP_1 contributed to only a small extent (6%) (Table 3). Nevertheless, the results show that EP_1 and EP_4 were functionally overexpressed in the respective cell lines and that functional EP_4 expression was higher than EP_1 expression in mono-transgenic cells as well as in the double-transgenic cell line.

Induction of IL-8 protein and mRNA synthesis and activation of the IL-8 promoter by PGE_2 in HEK-EP_1 but not in HEK or HEK-EP_4 cells

HEK, HEK- EP_1 and HEK- EP_4 cells were cultured for 20 h in the presence of 1 μM PGE_2 or 50 ng mL^{-1} TNFα. IL-8 protein in the supernatant of treated cells was quantified by ELISA, and the induction level of IL-8 mRNA was determined by real-time RT-PCR. In HEK and HEK-EP_4 cells PGE_2-stimulation did not change IL-8 protein or mRNA levels, whereas TNFα, a prototypical IL-8-inducing cytokine, induced IL-8 protein (HEK: 21-fold, HEK- EP_4 24-fold) and IL-8 mRNA (HEK: 20-fold, HEK- EP_4 21-fold) (Figure 1A and B). In both cell lines, TNFα induced IL-8 mRNA and protein synthesis to a comparable extent. By contrast, stimulation of HEK- EP_1 cells with PGE_2 induced a large increase in IL-8 protein (30-fold) and IL-8 mRNA (61-fold) levels that exceeded that induced by TNFα. An increase in IL-8 mRNA in HEK- EP_1 cells was also observed after stimulation with the EP_1-specific agonist ONO-

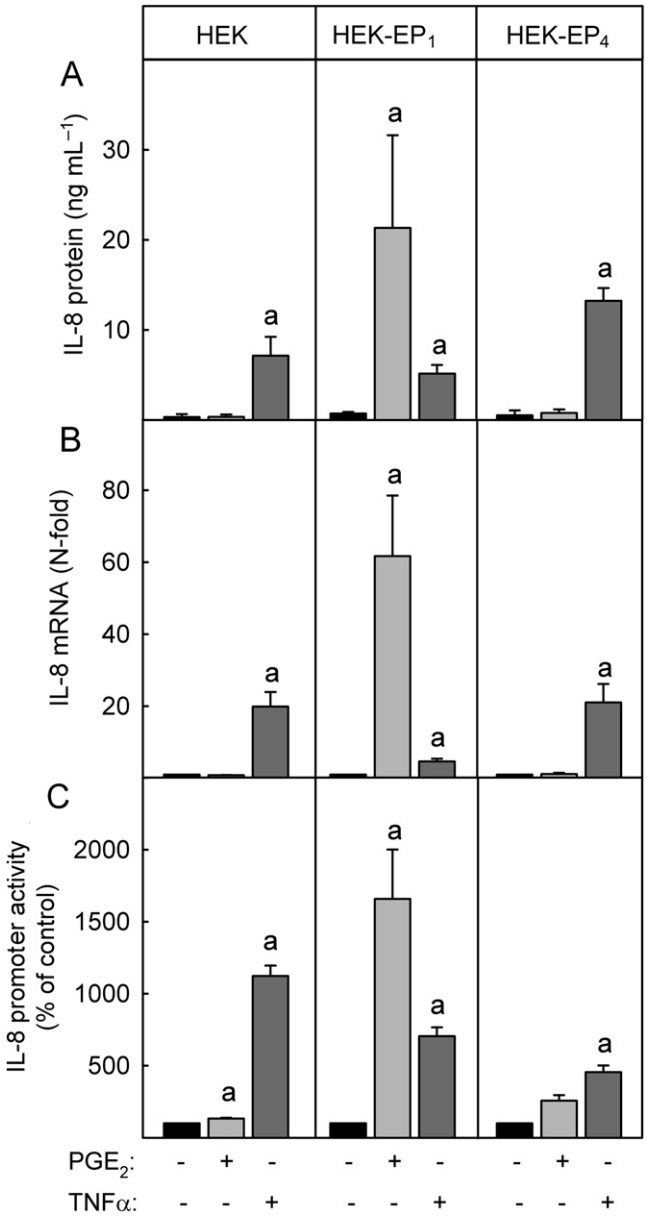

Figure 1

PGE_2 induces IL-8 protein and mRNA synthesis and IL-8 promoter activation in HEK-EP_1 but not in HEK or HEK-EP_4 cells. HEK, HEK-EP_1 and HEK-EP_4 cells were stimulated with 1 μM PGE_2 or 50 ng mL^{-1} TNFα for 20 h. For IL-8 promoter activation studies, cells were transfected with an IL-8 minimal promoter luciferase reporter gene plasmid before stimulation. (A) IL-8 protein: IL-8 released in the medium was measured by ELISA. Data shown are means ± SEM of three independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. a: significantly higher than control ($P < 0.05$). (B) IL-8 mRNA: IL-8 mRNA content was measured by qPCR as described in the Methods section with GAPDH as reference gene. Data shown are means ± SEM of four independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. a: significantly higher than control ($P < 0.05$). (C) IL-8 promoter activity: luciferase activity was measured in lysates as described in the Method section. Luciferase activity in control samples of each cell line was set at100%. Data shown are means ± SEM of three to six independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. a: significantly higher than control ($P < 0.05$).

DI-004, whereas the EP_4-specific agonist ONO-AE1-329 did not affect IL-8 expression in these cells (not shown). The TNFα-dependent increase was lower than in HEK and HEK-EP_4 cells (IL-8 protein: sixfold, IL-8 mRNA: fivefold). Thus, in stably transfected HEK cells, IL-8 synthesis was efficiently induced by PGE_2 via EP_1 but not via EP_4 receptors. To determine whether PGE_2-stimulated IL-8 mRNA transcription was a consequence of IL-8 promoter activation, HEK, HEK- EP_1 and HEK- EP_4 cells were transfected with a reporter gene construct expressing firefly luciferase under the control of an IL-8 promoter fragment. Sixteen hours after transfection, cells were treated with 1 μM PGE_2 or 50 ng mL^{-1} TNFα for 20 h. PGE_2 did not activate the IL-8 promoter in HEK cells. It activated the IL-8 promoter only slightly (but not statistically significant) in HEK- EP_4 cells (Figure 1C). By contrast, PGE_2 stimulation of HEK- EP_1 cells led to a pronounced IL-8 promoter activation (14-fold) that exceeded the activation by TNFα (ninefold), which also activated the IL-8 promoter in HEK (10-fold) and HEK- EP_4 cells (fivefold). These results show that despite the sensitivity for TNFα-dependent IL-8 promoter activation observed in all three cell lines, PGE_2-dependent IL-8 promoter activation, as well as IL-8 mRNA and protein induction was dependent on EP_1 expression in HEK- EP_1 cells.

Joint activation of EP_1 and EP_4 is essential for maximal PGE_2-stimulated IL-8 promoter activation and IL-8 mRNA and protein induction in HEK-EP_1 + EP_4 cells

Previous studies with human T-lymphocytes indicated a potential role for EP_4 in the regulation of IL-8 synthesis; however, PGE_2 did not by itself stimulate IL-8 synthesis in HEK cells expressing EP_4 receptors. Therefore, we investigated whether EP_4 receptors modulate the EP_1-dependent induction of IL-8 by PGE_2. Double transgenic HEK-EP_1 + EP_4 cells were created to analyse any potential crosstalk between both EP receptors. In HEK EP_1 + EP_4 cells, PGE_2 induced IL-8 production, IL-8 mRNA and IL-8 promoter activity. A significant induction was observed even after 10 h, whereas maximal activation was observed at around 20 h (Figure 2). In HEK-EP_1 + EP_4 cells, PGE_2 activates both receptors. At 20 h, it induced a 49-fold increase in IL-8 protein and a 39-fold increase in IL-8 mRNA (Figure 3A and B). Activation of EP_1 by an EP_1-specific agonist increased IL-8 mRNA and protein significantly but to a much lesser extent than PGE_2 (IL-8 protein: fourfold, IL-8 mRNA: 12-fold), whereas the EP_4-specific agonist only slightly, but not significantly, increased IL-8 (Figure 3A and B). A robust increase in IL-8 mRNA and protein, which reached the IL-8 mRNA levels stimulated by PGE_2, was achieved when HEK- EP_1 + EP_4 cells were stimulated with both receptor agonists together (IL-8 protein: 19-fold, IL-8 mRNA: 34-fold). These results show that the simultaneous activation of EP_1 and EP_4 receptors was necessary for maximal IL-8 induction by PGE_2. This could also be observed at the IL-8 promoter level. The EP_4 agonist activated the IL-8 promoter slightly but not significantly (3.2-fold), whereas stimulation with the EP_1 agonist led to a strong and significant activation of the IL-8 promoter (6.5-fold) but did not reach the activation levels observed in cells stimulated with PGE_2 (12-fold) (Figure 3C). Stimulation with both agonists

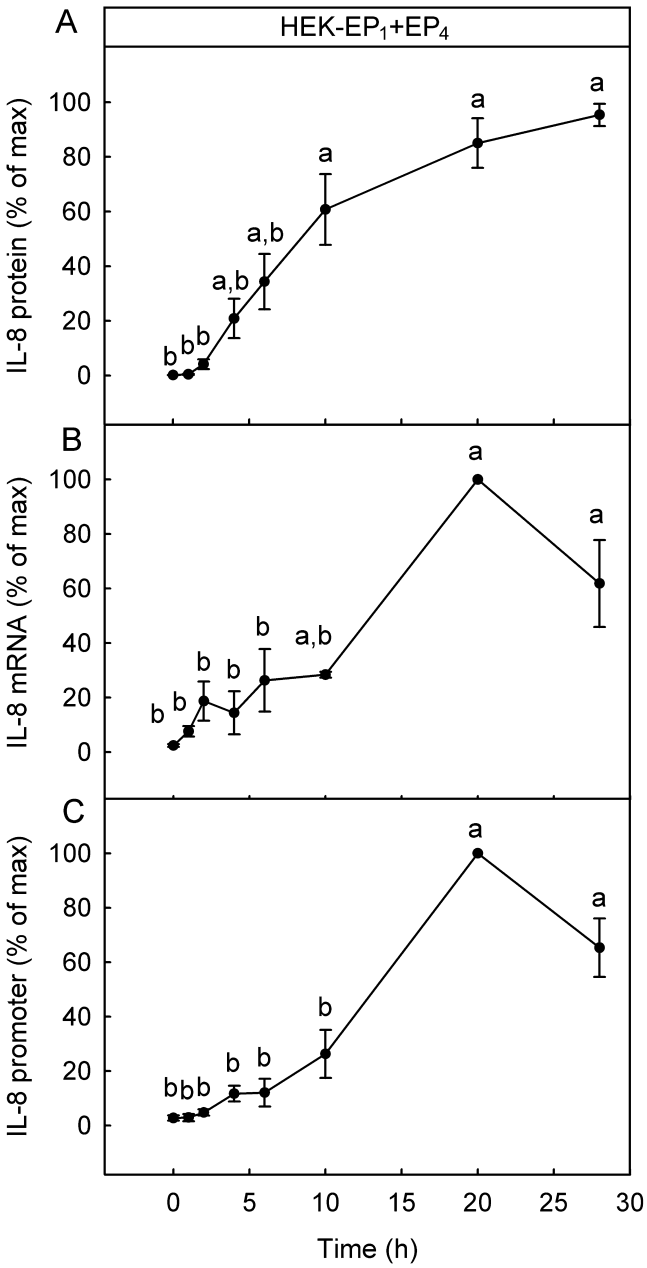

Figure 2

Time-dependence of PGE$_2$-induced IL-8 protein and mRNA synthesis and IL-8 promoter activation in HEK-EP$_1$ + EP$_4$ cells. HEK-EP$_1$ + EP$_4$ cells were stimulated with 1 μM PGE$_2$ for the times indicated. For IL-8 promoter activation studies, cells were transfected with an IL-8 minimal promoter luciferase reporter gene plasmid before stimulation. (A) IL-8 protein: IL-8 released into the medium was measured by ELISA. (B) IL-8 mRNA: IL-8 mRNA content was measured by qPCR as described in the Methods section with GAPDH as reference gene. (C) IL-8 promoter activity: luciferase activity was measured in lysates as described in the Methods section. Luciferase activity in samples of cells treated with PGE$_2$ for 20 h was set at100%. Data shown are means ± SEM of five independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. a: significantly higher than control; b: significantly lower than 20 h PGE$_2$ ($P < 0.05$).

together activated the IL-8 promoter to the same extent as PGE$_2$ (11-fold). The results show that for maximal activation of the IL-8 promoter and maximal PGE$_2$-mediated induction of IL-8 expression both EP$_1$ and EP$_4$ signal chains needed to be activated.

PGE$_2$-stimulated IL-8 promoter activation and IL-8 mRNA induction in HEK-EP$_1$ + EP$_4$ cells depends on NF-κB activation

Induction of IL-8 transcription by most stimuli is controlled by the transcription factor NF-κB. NF-κB activation is dependent on phosphorylation of I-κB by IKK complex, which directs I-κB to ubiquination and proteasomal degradation. To determine whether NF-κB activation is involved in PGE$_2$-stimulated IL-8 induction via EP$_1$ and EP$_4$ activation, HEK- EP$_1$ + EP$_4$ cells were treated with the IKK inhibitor BMS-34551 at the same time as stimulation with PGE$_2$. BMS-34551 had no effect on the basal IL-8 mRNA level and IL-8 promoter activity in these cells but completely blocked PGE$_2$-mediated IL-8 mRNA induction and IL-8 promoter activation (Figure 4A and B). This shows that PGE$_2$-stimulated IL-8 induction was dependent on NF-κB activation in HEK- EP$_1$ + EP$_4$ cells.

NF-κB activation by PGE$_2$-binding to EP$_1$ and EP$_4$

As PGE$_2$-mediated IL-8 promoter activation and IL-8 mRNA induction in HEK- EP$_1$ + EP$_4$ cells was dependent on IKK activation, NF-κB activation by PGE$_2$ was analysed in HEK, HEK- EP$_1$, HEK- EP$_4$ and HEK- EP$_1$ + EP$_4$-cells. To this end, cells were transfected with a NF-κB-luciferase reporter gene plasmid that encodes firefly luciferase under the transcriptional control of an artificial promoter containing multiple NF-κB binding sites. NF-κB activation was quantified by measurement of luciferase activity in extracts from cells stimulated with PGE$_2$ or TNFα for 20 h. PGE$_2$ did not activate NF-κB in HEK cells (Figure 5A). By contrast stimulation of HEK cells with TNFα (which activates NF-κB via TNFα-receptor 2 subtype signal chain) led to a sustained NF-κB activation (39-fold). In contrast to parental HEK cells, PGE$_2$ activated NF-κB reporter activity in HEK- EP$_1$ cells (13.5-fold). TNFα activated NF-κB reporter activity in HEK- EP$_1$ cells 17.9-fold, similar to the activation level induced by PGE$_2$ (Figure 5A). Stimulation of HEK- EP$_4$ cells with PGE$_2$ activated NF-κB only slightly (threefold), whereas TNFα led to the same strong NF-κB activation as in untransfected HEK cells (40-fold). Together, the experiments show that PGE$_2$ can activate NF-κB via EP$_1$ and less efficiently via EP$_4$. This is in contrast to results obtained in HEK-EP$_1$ + EP$_4$ cells expressing both receptors, where EP$_1$ and EP$_4$ specific agonists activated NF-κB reporter activity to a comparable extent (EP$_1$ agonist 10-fold and EP$_4$ agonist 7.8-fold; Figure 5B). When cells were stimulated with both agonists at the same time, NF-κB-activation was similar (21-fold) to the activation obtained after PGE$_2$-stimulation (26-fold). These results led to the hypothesis that simultaneous stimulation of EP$_1$ and EP$_4$ led to maximal NF-κB activation, which causes maximal PGE$_2$-dependent IL-8 promoter activation as well as IL-8 mRNA and protein induction.

To further corroborate the hypothesis that activation of EP$_1$ and EP$_4$ resulted in a direct activation of NF-κB, the phosphorylation and hence activation of the upstream

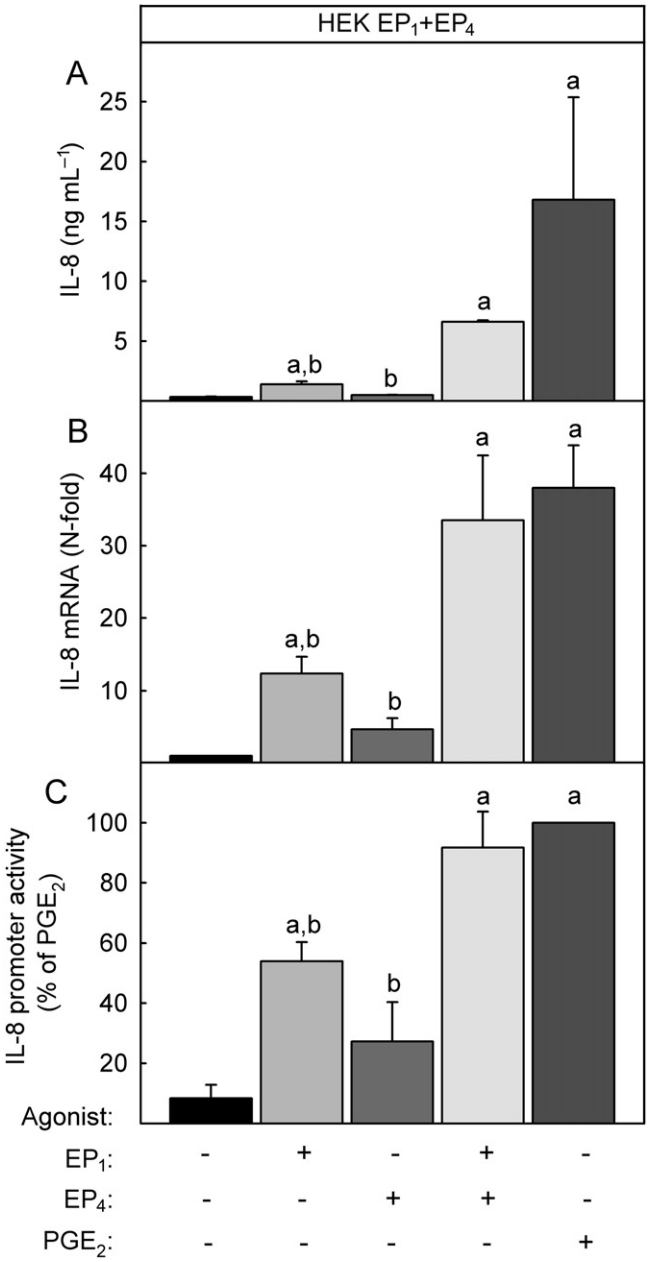

Figure 3

Simultaneous activation of EP_1 and EP_4 is essential for maximal PGE_2-stimulated IL-8 promoter activation and IL-8 mRNA and protein induction in HEK-EP_1 + EP_4 cells. HEK-EP_1 + EP_4 cells were stimulated with 1 μM PGE_2 or 1 μM of the EP receptor specific agonists ONO-DI-004 (EP_1) or ONO-AE1-329 (EP_4) or a combination of both agonists for 20 h. For IL-8 promoter activation studies, cells were transfected with an IL-8 minimal promoter luciferase reporter gene plasmid before stimulation. (A) IL-8 protein: IL-8 released in the medium was measured by ELISA. Data shown are means ± SEM of three independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. a: significantly higher than control and b: significantly lower than PGE_2 ($P < 0.05$). (B) IL-8 mRNA: IL-8 mRNA content was measured by qPCR as described in the Methods section with GAPDH as reference gene. Data shown are means ± SEM of five independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. a: significantly higher than control and b: significantly lower than PGE_2 ($P < 0.05$). (C) IL-8 promoter activity: luciferase activity was measured in lysates as described in the Methods section. Luciferase activity in control samples of each cell line was set at100%. Data shown are means ± SEM of three to six independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. a: significantly higher than control and b: significantly lower than PGE_2 ($P < 0.05$).

kinase, IKK was analysed by employing phosphospecific antibodies. Stimulation of HEK-EP_1 + EP_4 cells with PGE_2 resulted in a rapid and sustained phosphorylation of IKK (Figure 6A); phosphorylation was observed after 5 min and the enzyme remained phosphorylated over the subsequent 30 min. In HEK- EP_1 + EP_4 cells, the receptor subtype specific agonists both increased IKK phosphorylation slightly albeit not significantly (Figure 6B). Only simultaneous stimulation of the cells with both agonists resulted in significant IKK phosphorylation that was similar to that observed after stimulation with PGE_2 (Figure 6B).

NF-κB was activated by different EP_1/EP_4 signal chains

EP_1 + EP_4 are both GPCRs. The mechanism linking PGE_2 stimulation to NF-κB activation is currently unknown. To analyse the signal chains involved in NF-κB activation HEK-EP_1 cells and HEK- EP_1 + EP_4 cells were transfected with the NF-κB reporter gene plasmid and treated with inhibitors of intracellular signal chains along with PGE_2, EP receptor agonists or TNFα for 20 h before luciferase activity was measured. EP_1 is coupled to an increase in intracellular calcium concentration via activation of the $InsP_3$ receptor and/or modulating calcium channels (Katoh *et al.*, 1995; Ji *et al.*, 2010). The PLC inhibitor U73122 as well as the Ca^{2+}-chelator EGTA and the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-62 significantly reduced PGE_2-dependent NF-κB activation in HEK-EP_1 cells but did not prevent activation by TNFα (Figure 7). Inhibition of PKC with the PKC inhibitor bisindolylmaleimide (BIM) had no effect on PGE_2 or TNFα-mediated NF-κB activation in HEK-EP_1 cells. In contrast, NF-κB activation with the PKC activator PMA was completely inhibited after treatment with BIM (data not shown). These results show that activation of PLC as well as Ca^{2+}-signalling, but not PKC, mediate PGE_2-stimulated NF-κB activation by EP_1. Another signal molecule, which can phosphorylate and therefore activate IKK/NF-κB, is the tyrosine kinase Src. As Src can be activated by Ca^{2+}-signalling via CaMKII, the function of Src in EP_1-mediated NF-κB activation was analysed. As shown in Figure 7, Src inhibition by the inhibitor PP2 attenuated EP_1 but not TNFα receptor-mediated NF-κB activation. This confirms the involvement of Src in EP_1-stimulated NF-κB activation.

EP_4 couples to cAMP increase via Gs. In addition, activated EP_4 can form a complex with β-arrestin, which leads to activation of Src and subsequent activation of PI3K and Akt kinase by transactivation of the EGF receptor (Buchanan *et al.*, 2006). To find out which EP_4 signal chains are involved in PGE_2-mediated NF-κB-activation, HEK-EP_1 + EP_4 cells were treated with the PKA inhibitor H89, the Src inhibitor PP2 or the PI3K inhibitor wortmannin before stimulation with PGE_2 or EP receptor-specific agonists. Neither the PKA inhibitor

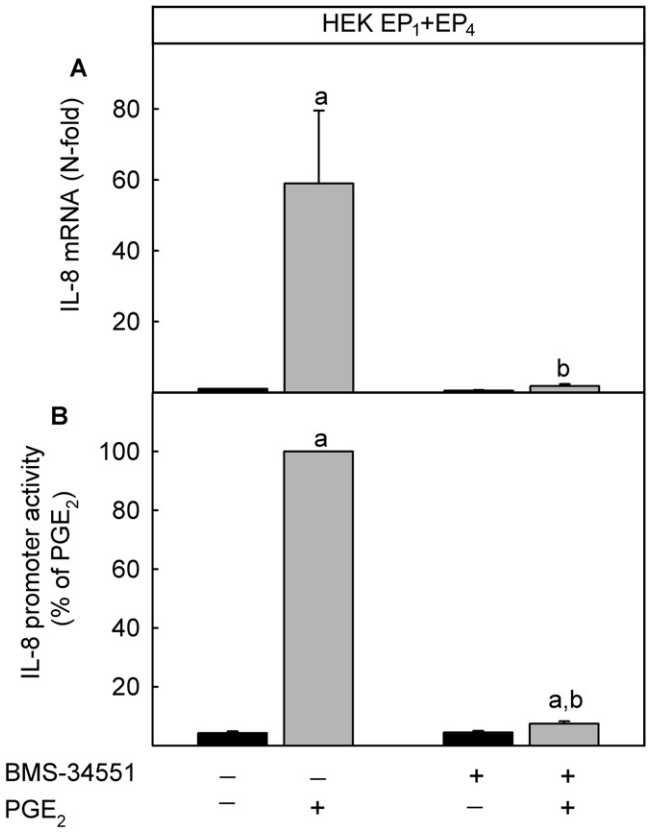

Figure 4

PGE_2-stimulated IL-8 promoter activation and IL-8 mRNA induction in HEK-EP_1 + EP_4 cells depend on NF-κB activation. HEK-EP_1 + EP_4 cells were stimulated with 1 μM PGE_2 in the absence or presence of the I-κB inhibitor BMS-34551 (10 μM) for 20 h. For IL-8 promoter activation studies, cells were transfected with an IL-8 minimal promoter luciferase reporter gene plasmid before stimulation. (A) IL-8 mRNA: IL-8 mRNA content was measured by qPCR as described in the Methods section with GAPDH as reference gene. Data shown are means ± SEM of five independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. a: significantly higher than control and b: significant lower than PGE_2 ($P < 0.05$). (B) IL-8 promoter activity: luciferase activity was measured in lysates as described in the Methods section. Luciferase activity in control samples of each cell line was set at100%. Data shown are means ± SEM of three to six independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. a: significantly higher than control and b: significantly lower than PGE_2 ($P < 0.05$).

H89 nor the PI3K inhibitor wortmannin affected NF-κB activation by PGE_2 or EP receptor-specific agonists (Figure 8). In contrast, treatment of the cells with the Src inhibitor PP2 significantly reduced NF-κB activation by PGE_2 and both EP_1 + EP_4 specific agonists (Figure 8), whereas PP2 did not affect TNFα-induced NF-κB activation (results not shown). This showed that EP_4-mediated activation of NF-κB was not PKA- or PI3K-dependent but was dependent on Src, which is also involved in NF-κB activation by EP_1. PP2 also inhibited the PGE_2-dependent phosphorylation and activation of IKK upstream of the NF-κB activation and the PGE_2-dependent induction of IL-8 mRNA downstream of the NF-κB activation (Figure 9A and B).

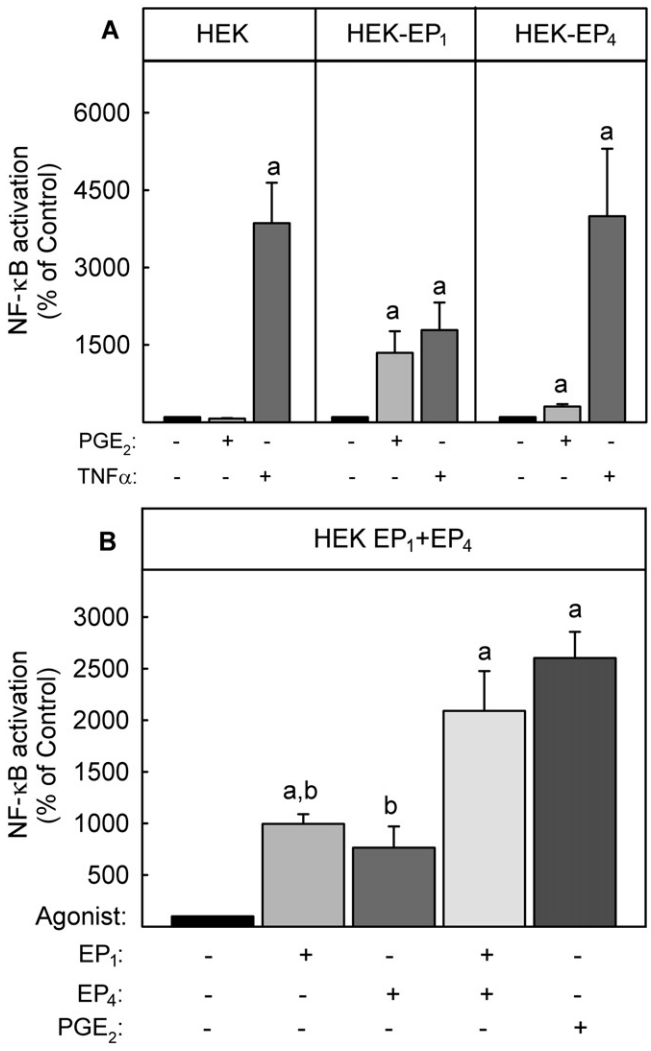

Figure 5

PGE_2 binding to EP_1 and EP_4 leads to NF-κB activation. HEK, HEK-EP_1 and HEK-EP_4 cells (A) as well as HEK-EP_1 + EP_4 cells (B) were transfected with a reporter gene plasmid with firefly luciferase under the control of multiple NF-κB binding sites. After 20 h, cells were stimulated with 1 μM PGE_2, 50 ng mL^{-1} TNFα or 1 μM of EP_1 and EP_4-specific agonists for a further 20 h. Luciferase activity was measured in lysates as described in the Methods section. Luciferase activity in control samples of each cell line was set at100%. Data shown are means ± SEM of three to six independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. a: significantly higher than control and b: significantly lower than PGE_2 ($P < 0.05$).

To further analyse the involvement of Src, the activation of Src was determined with antibodies against phospho tyrosine 416, which is phosphorylated upon activation of the enzyme and hence is a marker of Src activation. Although treatment of HEK-EP_1 + EP_4 cells with either EP_1 or EP_4 agonists resulted in a slight but non-significant phosphorylation of Src, the combined administration of EP_1 + EP_4 agonists or stimulation with PGE_2, which activates both receptor subtypes, resulted in similar and significant phosphorylation of Src (Figure 9C).

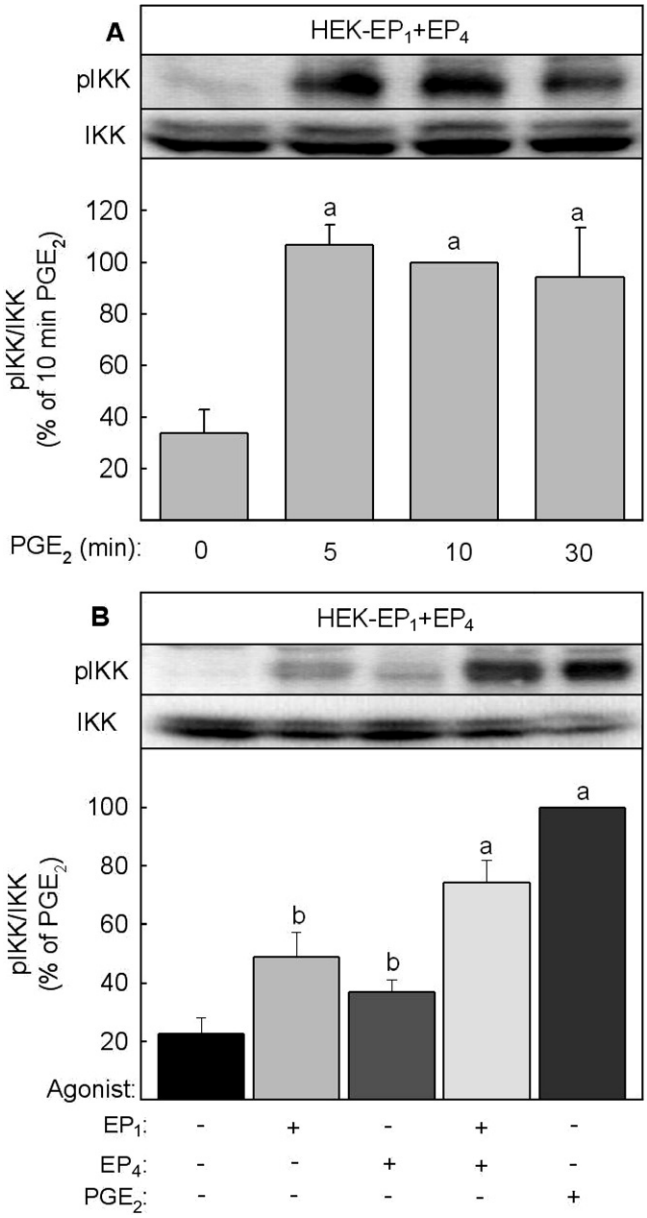

Figure 6

Requirement of simultaneous activation of EP_1 and EP_4 for maximal short-term activation of I-κB kinase (IKK) in HEK-EP_1 + EP_4 cells. HEK-EP_1 + EP_4 cells were incubated with 1 μM PGE_2 for the times indicated (A) or with 1 μM PGE_2 or receptor-specific agonists for 10 min (B). Proteins were extracted from cells with SDS sample buffer containing fluoride and vanadate to inhibit phosphatases. Phosphorylated and total IKKs were determined by Western blot using specific antibodies, peroxidase-coupled secondary antibodies and a luminogenic substrate. Band intensity was quantified luminometrically and expressed as ratio between phosphorylated and total protein. Values are means ± SEM of a minimum of three independent experiments. Statistics: Student's *t*-test for unpaired samples a: significantly higher than unstimulated control cells; b: significantly lower than cells stimulated with PGE_2, $P < 0.05$. Representative blots are shown.

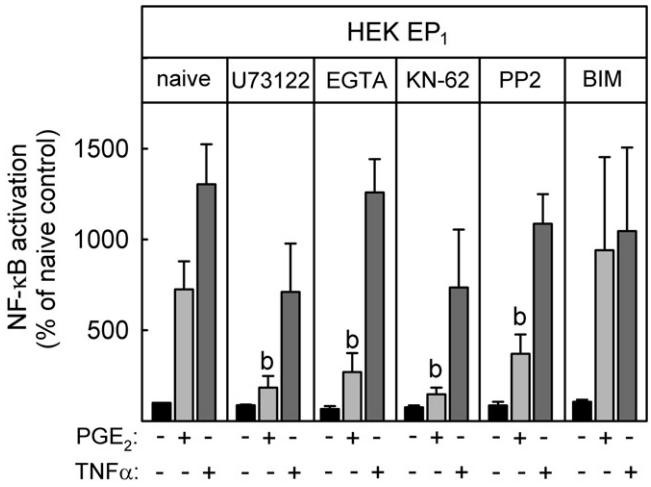

Figure 7

NF-κB activation by EP_1 is dependent on PLC, Ca^{2+} signalling and Src but not on PKC. HEK-EP_1 cells were transfected with a reporter gene plasmid with firefly luciferase under the control of multiple NF-κB binding sites. After 20 h, cells were stimulated with 1 μM PGE_2 or 50 ng mL^{-1} TNFα in the absence or presence or of the PLC inhibitor U73122 (10 μM), the Ca^{2+} chelator EGTA (2 mM), the CaMKII inhibitor KN-62 (10 μM), the Src kinase inhibitor PP2 (10 μM) or the PKC inhibitor BIM (0.15 μM) for a further 16 h. Luciferase activity was measured in lysates as described in the Methods section. Luciferase activity in control samples of each cell line was set at 100%. Data shown are means ± SEM of three to six independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. b: significantly lower than naive ($P < 0.05$).

Discussion

The results of the current study show that simultaneous activation of EP_1 + EP_4 signal chains was necessary for PGE_2-induced maximal activation of IL-8 in HEK293 cells. Although EP_1 activation alone was sufficient to induce a significant increase in IL-8, additional activation of EP_4 enhanced the level of IL-8 induced but did not affect IL-8 expression by itself. In contrast to studies describing PGE_2-enhanced IL-8 formation in T-lymphocytes activated by CD3/CD28 antibodies (Caristi *et al.*, 2005), which were independent of NF-κB but dependent on the transcription factor C/EBP homologues protein (CHOP), PGE_2-triggered IL-8 formation in the current study was NF-κB dependent.

Role of EP receptors in PGE_2-stimulated IL-8 induction

Inflammation is characterized by the infiltration of neutrophils, macrophages and lymphocytes into the injured tissue. The chemokine IL-8 is a potent chemoattractant for neutrophils and leukocytes. It was shown that IL-8 is elevated in a number of inflammatory diseases like asthma (Gibson *et al.*, 2001), colitis (Uguccioni *et al.*, 1999) and rheumatoid arthritis (Hwang *et al.*, 2004). IL-8 has been shown to be induced by many different stimuli. TNFα and IL-1β are known as highly potent stimulators of NF-κB-dependent IL-8 expression in various cell types. In addition to IL-8, TNFα and

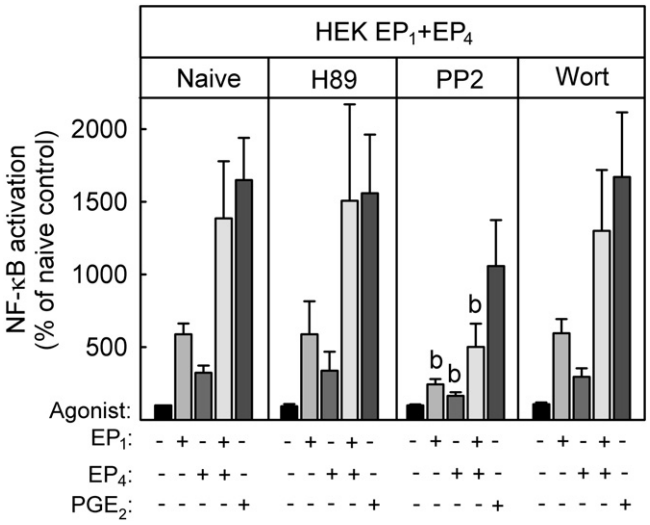

Figure 8

NF-κB activation by EP_1 and EP_4 is dependent on Src but not on PKA or PI3K. HEK-EP_1 + EP_4 cells were transfected with a reporter gene plasmid with firefly luciferase under the control of multiple NF-κB binding sites. After 20 h, cells were stimulated with 1 μM PGE_2 or EP_1/EP_4-specific agonists in the presence of the Src inhibitor PP2 (10 μM), the PKA inhibitor H89 (10 μM) or the PI3K inhibitor wortmannin (Wort; 0.1 μM) for a further 20 h. Luciferase activity was measured in lysates as described in the Methods section. Luciferase activity in control samples of each cell line was set at 100%. Data shown are means ± SEM of three to six independent experiments performed in triplicate. Statistics: Student's *t*-test for unpaired samples. b: significantly lower than naive ($P < 0.05$).

IL-1β induce COX-2, the key regulatory enzyme in prostanoid synthesis from arachidonic acid (Vlahos and Stewart, 1999). As a consequence, the concentration of prostaglandins, mainly PGE_2, is elevated in inflamed tissues. The actions of PGE_2 are mediated by its binding to four different GPCRs, EP_1-EP_4, which activate different G proteins and signal chains. EP_1 couples to Gq and Ca^{2+}-signalling (Ji *et al.*, 2010) whereas EP_2 and EP_4 couple to Gs and EP_3 to Gi (Breyer *et al.*, 2001). The role of PGE_2 in inflammation is controversial. A number of studies have demonstrated anti-inflammatory actions of PGE_2 including suppression of T-cell induction (van der Pouw Kraan *et al.*, 1995) and prevention of natural killer cell activation (Joshi *et al.*, 2001). In human macrophages PGE_2 suppresses LPS-induced formation of the chemokines IL-8, macrophage inflammatory protein (MIP)-1α, MIP-1β and monocyte chemotactic protein-1 by binding to EP_4 (Takayama *et al.*, 2002). In addition, PGE_2 suppresses TNFα-formation in mouse macrophages in a PKA-dependent manner and inhibits LPS-induced TNFα-formation in mouse Kupffer cells via Gs-coupled EP_2 and EP_4 receptors (Fennekohl *et al.*, 2002; Wall *et al.*, 2009).

In contrast, PGE_2 has been shown to stimulate IL-8 formation in human T-lymphocytes (Caristi *et al.*, 2005), cystic fibrosis airway epithelia cells (Vij *et al.*, 2008) and human colonic epithelial cells (Dey and Chadee, 2008). Interestingly, PGE_2 was also shown to be involved in IL-8 formation induced by the peptide hormone bradykinin in human airway smooth muscle cells. In these cells, the COX inhibitor indomethacin inhibited bradykinin-stimulated IL-8 formation, whereas exogenous PGE_2 activated the IL-8 promoter and enhanced IL-8 formation (Zhu *et al.*, 2003). In the present study, PGE_2 induced IL-8 formation only in EP_1 but not in EP_4 expressing cells. In HEK-EP_1 + EP_4, stimulation with a specific EP_1 agonist but not an EP_4 agonist activated IL-8 formation, whereas activation of both receptors was necessary for maximal IL-8 formation. The role of the EP_4 receptor, therefore, seems to be to enhance IL-8 formation triggered by EP_1 signal chains rather than to directly activate the induction of IL-8. These results are in line with experiments in T-lymphocytes where activation of both EP_1 and EP_4 was necessary for maximal PGE_2-induced IL-8 formation (Caristi *et al.*, 2005). In other cell types, EP_4 activation alone was found to be sufficient for PGE_2-mediated IL-8 induction. In Caco-2 cells overexpression of EP_4 but not of the EP_2 led to PGE_2-stimulated IL-8 formation (Dey *et al.*, 2009). In addition, stimulation of untransfected Caco-2 cells with an EP_4-specific agonist but not with an EP_2 agonist led to the same significant increase in IL-8 formation as stimulation with PGE_2. The fact that EP_4 activation induced IL-8 expression in Caco-2 but not in HEK293 cells may be due to activation of different signal chains by EP_4 in these cells. In addition to activation of the cAMP signal pathway, EP_4 was reported to signal via the Src-dependent EGF receptor transactivation and subsequent activation of PI3K (Buchanan *et al.*, 2006). Another possibility is that in Caco-2 but not in HEK293 cells PGE_2 may activate the release of IL-8-inducing mediators, whose IL-8-inducing effect may be enhanced by PGE_2-mediated EP_4 activation. Nevertheless, the results of our study clearly demonstrate that PGE_2-mediated IL-8 formation is directly induced by EP_1 signal chains in our experimental system. In contrast to EP_4, which is widely expressed throughout the body, EP_1 is expressed mainly in the colon and kidney. Therefore, PGE_2-stimulated IL-8 formation in EP_1/EP_4 expressing cells in these organs, which trigger inflammatory processes, is quite a likely paradigm.

Targets of EP_1 + EP_4-dependent signal chains in PGE_2-stimulated IL-8 formation

IL-8 expression is mainly regulated on the transcriptional level. A core IL-8 promoter region spanning nucleotides –1 to –133 is essential and sufficient for transcriptional regulation of the gene. The core promoter includes potential binding sides for the transcription factors AP-1, C/EBP and NF-κB (Hoffmann *et al.*, 2002). Whereas the NF-κB site is essential for IL-8 activation by various stimuli in most cell lines, the AP-1 and C/EBP sites are not required for primary induction but for maximal gene expression (Hoffmann *et al.*, 2002). In addition to these three transcription factors, IL-8 induction by PGE_2 in cystic fibrosis cells or T-Lymphocytes was mediated by activation of transcription factor CHOP (Caristi *et al.*, 2005). A CHOP responsive element is located between bases –130 and –137 in the IL-8 promoter, which overlaps with the AP-1 site. Surprisingly, in cystic fibrosis cells and T-cells, PGE_2-stimulated IL-8 formation was independent of NF-κB; the NF-κB inhibitor caffeic acid did not prevent PGE_2-stimulated IL-8 formation. It was also shown in these studies that CHOP binds to the IL-8 promoter after PGE_2 stimulation and that deletion of the CHOP responsive element inhibited PGE_2-stimulated activation of the IL-8 promoter. By contrast, the

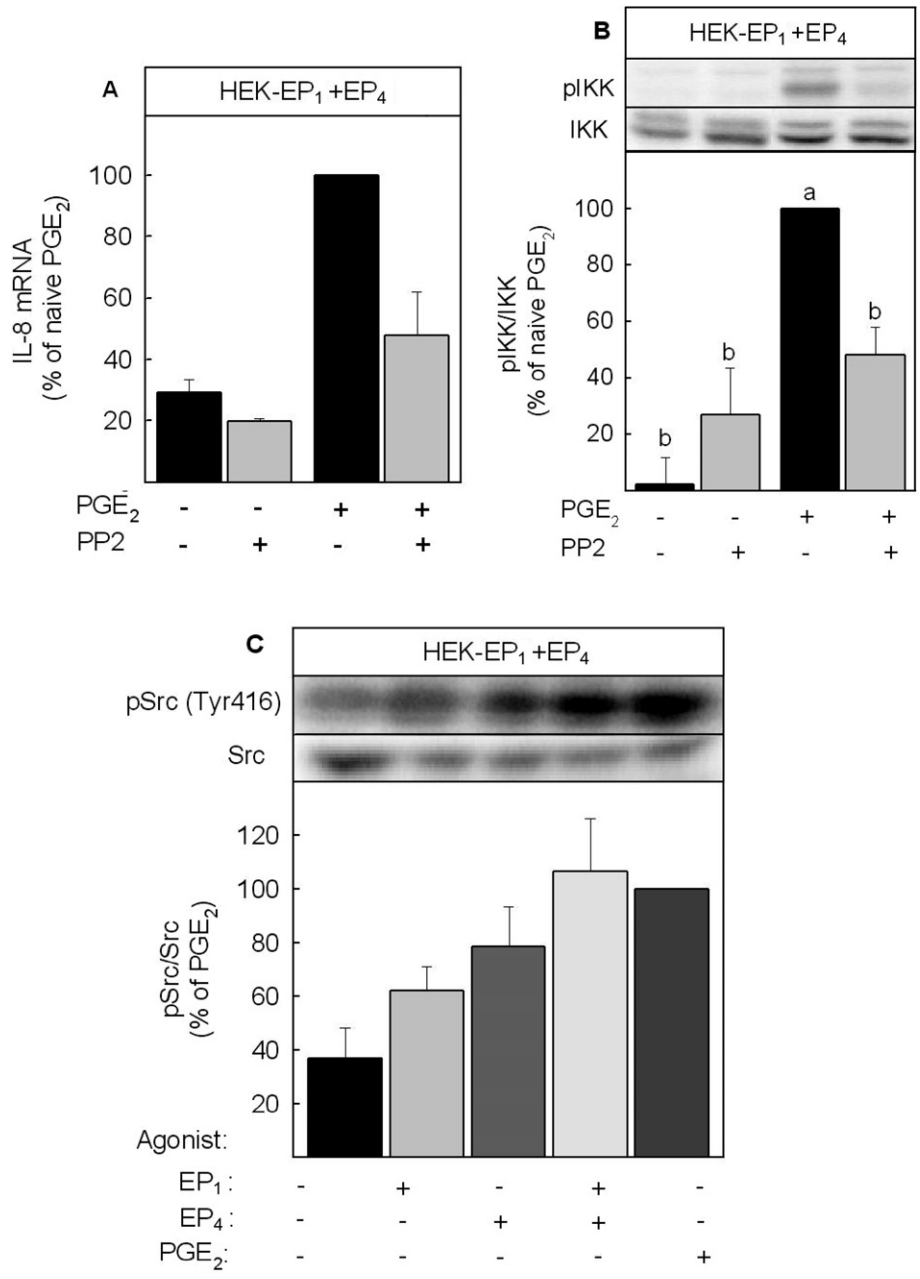

Figure 9

Involvement of PGE_2-mediated activation of Src kinase in HEK-EP_1 + EP_4 cells in PGE_2-dependent IL-8 induction. (A) HEK-EP_1 + EP_4 cells were stimulated with 1 μM PGE_2 in the absence or presence of the Src kinase inhibitor PP2 (10 μM) for 20 h. IL-8 mRNA content was measured by qPCR as described in the Methods section with GAPDH as reference gene. (B and C): HEK-EP_1 + EP_4 cells were pre-incubated with PP2 (10 μM) for 1 h and subsequently stimulated with 1 μM PGE_2 or receptor-specific agonists for 10 min. Proteins were extracted from cells with SDS sample buffer containing fluoride and vanadate to inhibit phosphatases. Phosphorylated and total IKKs (B) or Src kinase (C) were determined by Western blot using specific antibodies, peroxidase-coupled secondary antibodies and a luminogenic substrate. Band intensity was quantified luminometrically and expressed as ratio between phosphorylated and total protein. Values are means ± SEM of a minimum of three independent experiments. Statistics: Student's *t*-test for unpaired samples a: significantly higher than non-stimulated control cells; b: significant lower than cells stimulated with PGE_2, $P < 0.05$. Representative blots are shown.

results of the present study demonstrated that PGE_2-mediated IL-8 formation by activation of EP_1 + EP_4 were dependent on NF-κB activation. This conclusion is based on several lines of evidence. Firstly, PGE_2-stimulated activation of the IL-8 promoter as well as IL-8 mRNA induction was completely abolished by the IKK-inhibitor BMS-34551. Secondly, stimulation with PGE_2 increased NF-κB activity in HEK293 cells overexpressing EP_1 and/or EP_4 but not in untransfected cells. Thirdly, PGE_2 activation of NF-κB in HEK-EP_1, HEK-EP_4 and HEK-EP_1 + EP_4 cells has the same profile as PGE_2 activation of

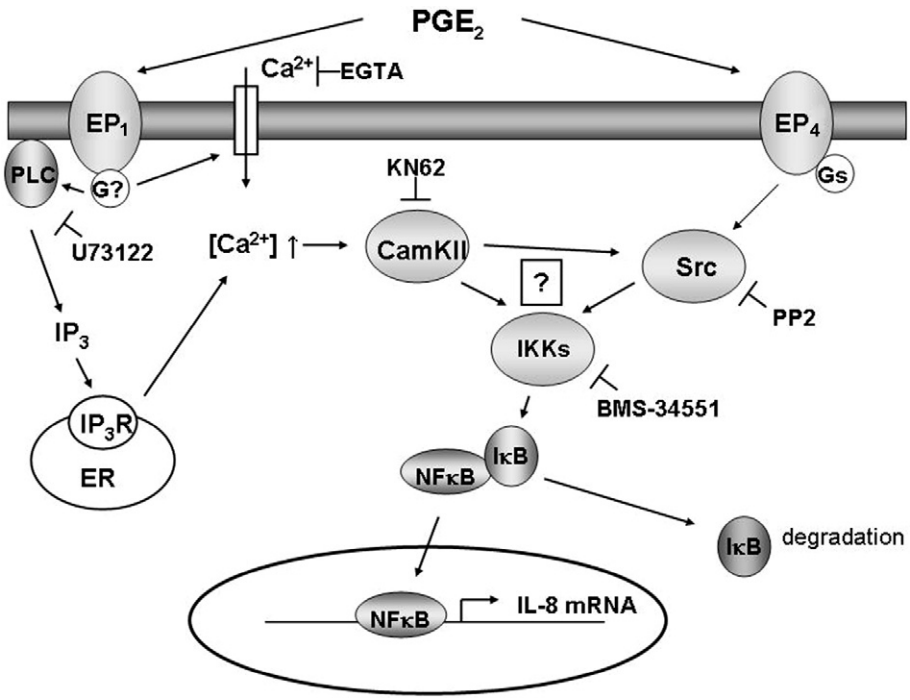

Figure 10

Model of PGE_2/EP_1 + EP_4-mediated NF-κB activation and IL-8 induction. PGE_2-bound EP_1 activates PLC and/or Ca^{2+}-channels in the plasma membrane by Gq and/or an as yet unknown G protein. This leads to a transient increase in intracellular Ca^{2+}, that thereby activates CamKII. CamKII directly or indirectly via tyrosine kinase Src phosphorylates IKKs and signals I-κB degradation. Src-mediated IKK-phosphorylation is also triggered by PGE_2-binding to the Gs-coupled EP_4, which leads to maximal I-κB degradation. The released NF-κB translocates to the nucleus and promotes IL-8 transcription by binding to the IL-8 promoter.

the IL-8 promoter, induction of IL-8 mRNA and IL-8 protein expression. NF-κB and IL-8 induction was predominantly activated by EP_1, whereas stimulation of EP_4 had only a minor effect on NF-κB activation and IL-8 formation. Maximal NF-κB activation as well as IL-8 induction was observed when both receptors were stimulated at the same time in HEK-EP_1 + EP_4 cells.

EP receptor signal chains leading to NF-κB activation

A number of GPCRs have been shown to activate NF-κB. They include receptors for adenosine (Liu and Wong, 2004), bradykinin (Xie *et al.*, 2000) and somatostatin (Liu and Wong, 2005). These receptors activate NF-κB via the regulation of Gi, Gq and Gq-related G proteins like G14 or G16. G protein coupling of EP_1 is controversial. Although EP_1 activation led to a robust increase in Ca^{2+} concentration, there was only a very modest increase in $InsP_3$ generation. It was, therefore, suggested that EP_1 is not coupled to Gq but to activation of Ca^{2+} channels via an as yet undefined G protein (Katoh *et al.*, 1995). However, Gq coupling of the EP_1 receptor in HEK cells has also been reported (Ji *et al.*, 2010). Our study suggests that both signal chains are involved in PGE_2-stimulated NF-κB activation by EP_1, because NF-κB activation was significantly inhibited by the PLCβ inhibitor U73122, the Ca^{2+}-chelator EGTA and the CaMKII inhibitor KN-62. Elevation of intracellular calcium concentration can activate IKK activity through the action of CaMKII. The CaMKII inhibitor KN-62 blocks IKK/NF-κB activation by the somatostatin receptor sst_2 (Liu and Wong, 2004); however, the exact mechanism by which CaMKII regulates IKK activity remains unclear. Compared to the role of CaMKII, activation of PKC was not involved in EP_1-mediated NF-κB activation because the specific PKC inhibitor BIM did not alter PGE_2-stimulated NF-κB activity. This differs from the regulation of NF-κB by sst_2 where PKC activation was necessary for agonist-mediated IKK/NF-κB activation. In contrast to the PKC-independent activation of NF-κB by PGE_2, NF-κB activation by the phorbol ester PMA in HEK-EP_1 cells was completely abolished by BIM, showing that PKC-dependent NF-κB activation occurs in these cells and that the inhibitor BIM was functional.

In addition to the identification of PLCβ and CaMKII as transducers of EP_1-mediated NF-κB activation, it was shown that inhibition of the tyrosine kinase Src significantly attenuated EP_1-mediated NF-κB activation in HEK-EP_1 and in HEK-EP_1 + EP_4 cells. Src inhibition also blocked EP_4 agonist-stimulated IKK phosphorylation, NF-κB activation and induction of IL-8 mRNA in HEK-EP_1 + EP_4 cells, whereas inhibitors of PKA and PI3K, which have been described as signal transduction targets activated by EP_4, were ineffective. These results indicate a central role for Src in PGE_2-stimulated NF-κB activation. Once activated, Src was shown to associate directly with the IKK complex, leading to IKK phosphorylation, I-κB degradation and NF-κB activation (Lee *et al.*, 2007). Both EP_1 and EP_4 have been demonstrated to be activators of

Src (Tang *et al.*, 2005; Dey *et al.*, 2009). Although activation of Src by EP_1 most likely occurred via activation of PLCβ and CaMKII, activation by EP_4 was dependent on the formation of an EP_4/β–arrestin/Src 'signalosome complex'. Because Src can be activated by both receptors via different signal chains leading to NF-κB activation, Src might be the signal molecule that links PGE_2-stimulated IL-8 formation induced by EP_1 and to that activated by EP_4stimulation (Figure 10). Overall, our data indicate that simultaneous stimulation of EP_1 and EP_4 receptors by PGE_2 induces a marked elevation in the expression of IL-8.

Acknowledgement

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Conflict of interest

No conflicts of interests to declare.

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