European Journal of Inflammation

Original Research Article

PGE_2 stimulates COX-2 expression via EP2/4 receptors and acts in synergy with IL-1β in human follicular dendritic cell-like cells

European Journal of Inflammation
Volume 16: 1–9

DOI: 10.1177/2058739218796386
journals.sagepub.com/home/eji
SAGE

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Abstract

PGE_2 is the major lipid mediator of inflammation produced by multiple cell types including follicular dendritic cells (FDCs) of the lymphoid tissue. We have investigated the immunoregulatory function of PGE_2 and its production mechanism using FDC-like cells isolated from human tonsil. Our recent observation of COX-2-inducing effect of PGE_2 prompted us to identify the responsible receptor in this study. Pharmacologic approaches were adopted and Western blotting was utilized to measure protein expression levels. Agonists selective for EP2 and EP4 significantly stimulated COX-2 expression, while antagonists for these receptors prevented PGE_2 from triggering COX-2 induction. The combined addition of EP2 and EP4 antagonists resulted in further inhibition of PGE_2. In contrast, EP1 and EP3 antagonists failed to exhibit the inhibitory effect on PGE_2-induced COX-2 expression. Since PGE_2 achieves COX-2 induction by repressing Akt activation in FDC-like cells, we confirmed EP2 and EP4 being the targets of PGE_2 by examining the effects of E-prostanoid (EP) agonists and antagonists on the level of Akt phosphorylation. After the identification of PGE_2 receptor, we examined the effect of PGE_2 on IL-1β-induced COX-2 expression. PGE_2 and IL-1β brought about a synergistic induction of COX-2 expression. Taken together, this study implies the impact of the combined role of eicosanoids and cytokines in inflammatory milieu.

Keywords

COX-2, EP receptor, follicular dendritic cell, PGE_2

Date received: 18 March 2018; accepted: 26 July 2018

Introduction

PGE_2 is the pivotal lipid mediator of inflammation produced from the inner cell membrane phospholipids by the sequential actions of phospholipases, cyclooxygenases (COXs), and PGE synthases (PGESs).[1] The major cellular source of PGE_2 includes infiltrating inflammatory cells, epithelial cells, and fibroblasts, although it is produced by all cell types of the body.[2] Follicular dendritic cell (FDC), a specialized stromal cell found in the primary and secondary follicles of the lymphoid tissue,[3] also produces PGE_2. Heinen et al.[4] reported the detection of PGE_2 in FDC-enriched cell fraction and Butch et al.[5] reported that indomethacin reverses FDC-mediated inhibition of T-cell proliferation. Our laboratory demonstrated that FDC indeed expresses

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COX-2 and PGES in situ.[6,7] PGE_2 inhibited T-cell proliferation and apoptosis[6] and enhanced germinal center B-cell survival.[8] PGE_2 exerts pleiotropic functions by binding to specific G protein–coupled receptors (EP1–EP4) on the target cell membrane.[2] Each E-prostanoid (EP) receptor is known to induce changes in second messenger molecules such as Ca^{2+}, cAMP, and inositol phosphates.[9] EP1 stimulation leads to intracellular Ca^{2+} release via phospholipase C and phosphoinositol turnover and EP2 and EP4 increase cAMP levels through adenylate cyclase, whereas EP3 triggering brings about various modulation of cAMP and Ca^{2+} levels depending on the responding cell types.[9–11]

To maximize the physiological relevance of in vitro observations for human subjects, we have utilized primary FDC-like cells to understand the regulation mechanisms of prostaglandin production. In a recent report, we demonstrated that PGE_2 induces COX-2 protein expression in FDC-like cells.[12] This study was undertaken to specify the EP receptor that transduces PGE_2 signal leading to COX-2 upregulation in this type of cells. We further examined the impact of PGE_2 on IL-1β-induced COX-2 expression.

Materials and methods

Antibodies and other reagents

Following antibodies were used in this study. Antibodies specific to COX-1 and COX-2 were purchased from Santa Cruz Biotechnology. Specific antibodies against phosphorylated Akt and total Akt were purchased from Cell Signaling Technology. Anti-β-actin antibody was obtained from Sigma-Aldrich. Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Southern Biotech), anti-rabbit IgG (Abcam), and anti-goat IgG (Koma Biotech). PGE_2, 6-keto-$PGF_1\alpha$, EP agonists (17-phenyl-ω-PGE_2, butaprost, sulprostone, and CAY10598 for EP1, EP2, EP3, and EP4, respectively), and EP antagonists (ONO-8711, PF-04418948, L-798,106, and GW627368 for EP1, EP2, EP3, and EP4, respectively) were purchased from Cayman Chemical. IL-1β was obtained from R&D Systems.

Preparation and culture of FDC-like cells

Human tonsils obtained from the routine tonsillectomy were handled in accordance with the approval and guidelines of Kangwon National University (2016-10-002-001). FDC-like cells are fibroblastic adherent cells obtained through the enzyme digestion of human tonsils as described previously.[13] The purity of cells was more than 98% as assessed by a FACSCalibur (BD Biosciences) for the expression of FDC-specific marker 3C8 as reported.[14] They proliferate under the conventional culture condition and display antigenic and functional features of FDC.[15] FDC-like cells are used until they exhibit degenerate features in culture. Cell culture media was RPMI-1640 media (Gibco) containing 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 100 U/mL penicillin G (Sigma-Aldrich), and 100 μg/mL streptomycin (Life Technologies).

Immunoblotting

Cells were rinsed with cold phosphate-buffered saline (PBS) and then lysed in Pro-Prep cell extract solution (Intron Biotechnology). Centrifugation of lysates at 15,000*g* for 10 min at 4°C was conducted to remove insoluble materials, followed by protein quantification using a BCA Protein Assay Kit (Thermo Scientific). Proteins in the lysates were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 3% nonfat-dried milk for 1 h and then treated with primary and secondary antibodies. The PVDF membranes were treated with chemiluminescent solution (Elpis Biotech) and exposed to X-ray film for visualization of the detected proteins.

Statistical analysis

All data were analyzed with GraphPad Prism 5.04 software to be presented as the mean ± SEM of at least three independent experiments. The statistical significance of differences was determined using Student's t test. $P < 0.05$ was considered significant.

Results

EP2 and EP4 receptors mediate PGE_2-stimulated COX-2 protein induction

In order to specify the EP receptor that mediates PGE_2-induced COX-2 protein expression in FDC-like cells, we first examined the effects of

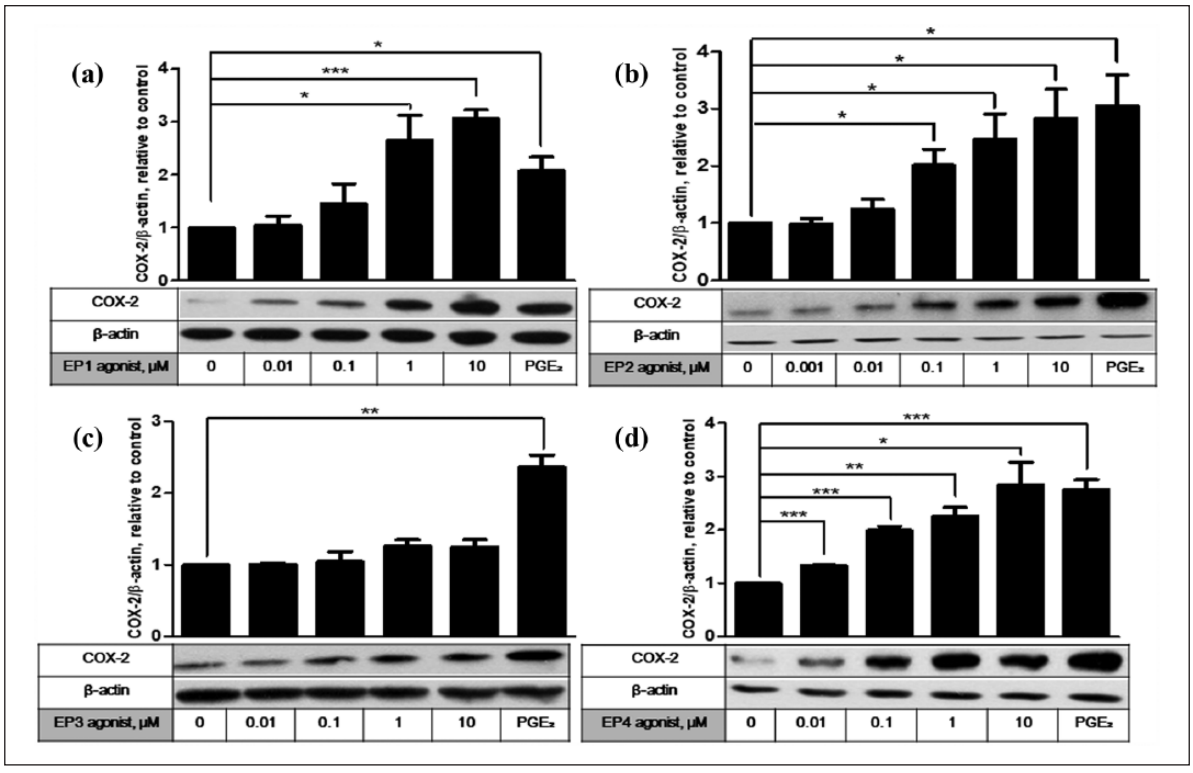

Figure 1. EP1, EP2, and EP4 agonists stimulate COX-2 expression in FDC-like cells. FDC-like cells (3×10^5 cells) were cultured in the indicated concentrations of agonists specific to (a) EP1, (b) EP2, (c) EP3, and (d) EP4 PGE_2 receptors as described in *Methods*, and then expression levels of COX-2 and β-actin were measured by the immunoblotting technique at the end of 4 h incubation. PGE_2 was included in the culture as a positive control of COX-2 expression at the concentration of 10 μM. The graphs show means ± SEM of three independent experiments with the representative immunoblots. The intensities of COX-2 bands were normalized by the levels of β-actin. Statistical significance was analyzed with Student's t test by comparing the values with the untreated control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

EP agonists on COX-2 protein expression. FDC-like cells were cultured in the presence of agonists specific to four types of PGE_2 receptors, EP1–EP4, and then COX-2 expression levels were measured by the immunoblotting technique at the end of 4-h culture. COX-2 levels induced by 10 μM PGE_2 were used as positive controls.[12] As shown in Figure 1, EP1-, EP2-, and EP4-selective agonists significantly increased COX-2 expression, which was concentration-dependent between 0.01 and 10 μM concentrations. At the concentration of 10 μM, these agonists yielded a threefold increase in COX-2 expression. In contrast, EP3 agonist failed to modulate COX-2 expression. Next, EP antagonists were employed to analyze their effects on PGE_2-mediated COX-2 expression. Antagonists were pre-treated to FDC-like cells 1 h before the addition of suboptimal amounts of PGE_2 (1 μM). All the antagonists alone did not modulate the background-level expression of COX-2 when incubated alone with FDC-like cells. However, specific inhibition was observed with EP2 and EP4 antagonists, which reduced COX-2 expression by 51% and 36% at the concentrations of 10 and 1 μM, respectively (Figure 2(b) and (d)). Although the inhibition obtained with each antagonist was partial, almost complete inhibition of PGE_2 activity was observed with the combined addition of EP2 and EP4 antagonists ($P = 0.0386$) (Figure 3). Different from EP1 agonist which enhanced COX-2 expression, EP1 antagonist did not prevent PGE_2 from the COX-2 induction between 0.1 and 10 μM concentrations (Figure 2(a)). In line with the results with EP3 agonist, EP3 antagonist did not modulate the COX-2 expression induced by PGE_2 (Figure 2(c)). The inhibitory effects of antagonists were specific to COX-2 since COX-1 expression was not affected as shown in Figure 2. Combined, these data imply that PGE_2 induces COX-2 protein expression through EP2 and EP4 receptors.

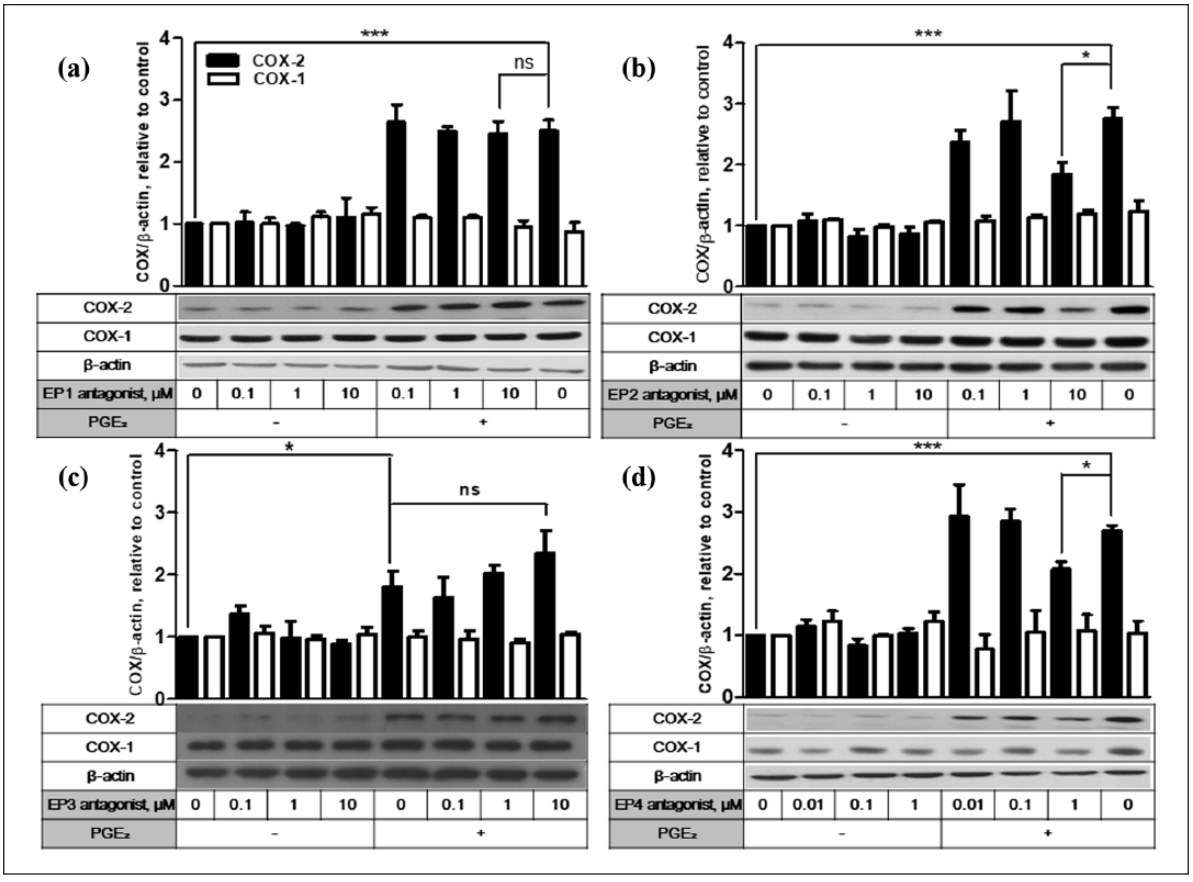

Figure 2. EP2 and EP4 antagonists hinder PGE_2 from stimulating COX-2 expression in FDC-like cells. FDC-like cells (3×10^5 cells) were pre-treated with the indicated concentrations of antagonists specific to (a) EP1, (b) EP2, (c) EP3, and (d) EP4 PGE_2 receptors as described in *Methods*, 1 h before the addition of PGE_2 (1 μM). At the end of 4 h culture, the immunoblotting technique was conducted to measure expression levels of COX-1, COX-2, and β-actin. The graphs show means ± SEM of three independent experiments with the representative immunoblots. The intensities of and COX-1 and COX-2 bands were normalized by the levels of β-actin. One-way ANOVA with Tukey's multiple comparison test reveals the overall significant difference, and the intergroup significance levels are noted in the figure (*$P < 0.05$; ***$P < 0.001$; ns, non-significant).

The inhibitory effect of PGE_2 on Akt phosphorylation is mediated by EP2 and EP4 receptors

We have recently demonstrated that COX-2 induction by PGE_2 is achieved by inhibiting Akt phosphorylation in FDC-like cells.[12] Paying attention to this finding, we envisioned that the effects of EP agonists and antagonists on Akt phosphorylation might be examined as a confirmatory approach of this study. As shown in Figure 4(a), FDC-like cells displayed high levels of Akt phosphorylation before treatment with EP agonists. Stimulation with graded concentrations of EP1 agonist for 30 min led to a dose-dependent decrease of Akt phosphorylation. A significant decrease of Akt phosphorylation was obtained with EP1 agonist from 0.1 μM concentration, reaching its optimal inhibition at 10 μM, which was comparable to that obtained with PGE_2. The EP1 agonist did not modulate total Akt protein expression levels during this culture period. Similar results were procured with EP2 and EP4 agonists (Figure 4(b) and (d)). In contrast, EP3 agonist did not affect the phosphorylation levels. Finally, the impact of antagonists on Akt phosphorylation levels was examined. Culture with suboptimal concentration of PGE_2, 1 μM, resulted in a significant inhibition of Akt phosphorylation, which was reversed by 10 μM of EP2 or EP4 antagonists but not by EP1 or EP3 antagonists (Figure 5). All together, the results with EP agonists and antagonists regarding the Akt phosphorylation corroborated those on COX-2 expression.

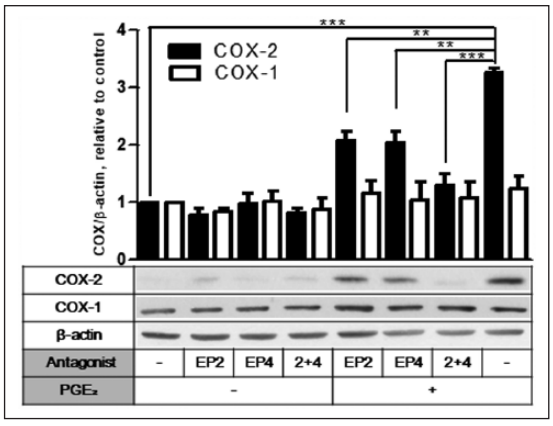

Figure 3. The combined addition of EP2 and EP4 antagonists results in further inhibition of PGE_2-induced COX-2 expression. EP2 and EP4 antagonists were added alone or together to FDC-like cells (3×10^5 cells) for 1 h before the further incubation in the presence or absence of PGE_2 (1 μM) for 4 h. COX-1, COX-2, and β-actin expression levels were measured by the immunoblotting technique. The representative immunoblots out of three reproducible experiments and the statistical analysis data (means ± SEM) are shown. One-way ANOVA with Tukey's multiple comparison test reveals the overall significant difference, and the intergroup significance levels are noted in the figure (**$P < 0.01$; ***$P < 0.001$).

PGE_2 augments IL-1β-induced COX-2 expression

The identification of PGE_2 receptor prompted us to examine the effect of PGE_2 on IL-1β-induced COX-2 expression in FDC-like cells. IL-1β stimulates COX-2 expression of FDC-like cells in a dose-dependent fashion.[16] Since PGE_2 also stimulates COX-2 expression, a suboptimal dose of IL-1β, 1 pg/mL, was used to determine the impact of co-treatment. As shown in Figure 6, PGE_2 acted in synergy with IL-1β in the stimulation of COX-2. The synergistic effect of PGE_2 was specific to COX-2 because COX-1 protein expression was not modulated by the combined addition. Next, we examined the specificity of PGE_2 activity by comparing the effect of PGE_2 with that of 6-keto-$PGF_1\alpha$, a stable hydrolyzed metabolite of PGI_2. Interestingly, 6-keto-$PGF_1\alpha$ displayed the synergistic effect with IL-1β, although it failed to enhance COX-2 expression when added alone to FDC-like cells (Figure 6(b)). Similar results were obtained with another stable metabolite of arachidonic acid, thromboxane B_2 (data not shown).

Discussion

The aim of this study was to understand the molecular mechanism of PGE_2 in COX-2 production and further its significance using FDC-like cells. Our conclusion that PGE_2 induces COX-2 expression via EP2 and EP4 receptors was obtained from the following results: (1) EP2 and EP4 agonists significantly enhanced COX-2 expression, (2) EP2 and EP4 antagonists prevented PGE_2 from upregulating COX-2, (3) the combined addition of EP2 and EP4 antagonists resulted in further inhibition of PGE_2-triggered COX-2 induction, (4) EP2 and EP4 agonists reduced Akt phosphorylation, and (5) EP2 and EP4 antagonists significantly reversed PGE_2-induced downregulation of Akt phosphorylation. In support of our conclusion, we verified the messenger RNA (mRNA) expression of EP2 and EP4 in FDC-like cells (data not shown). Although EP1 agonist stimulated COX-2 expression and reduced Akt phosphorylation, implying that the COX-2 induction and inhibition of Akt phosphorylation might be obtained by cytoplasmic Ca^{2+} increase via EP1 as well as cAMP engagement via EP2 or EP4, we excluded EP1 from the target receptors of PGE_2 in this cell type because EP1 antagonist failed to antagonize the effects of PGE_2 on COX-2 expression and Akt phosphorylation. Similar to our results, PGE_2 regulates cytokine production in synovial fibroblasts[11] and in plasmacytoid dendritic cells[17] and Th17 cell differentiation[18] through EP2 and EP4 receptor signaling. In addition, PGE_2-EP2/EP4 signaling mediates joint inflammation in mouse collagen–induced arthritis.[19] In contrast, PGE_2 stimulates CD95 ligand expression in colon cancer cells via EP1[20] and exacerbates murine arterial thrombosis via platelet EP3.[21] In addition, PGE_2 induces PGE_2 synthesis in HCA-7 colon cancer cells[22] and interleukin-8 production in pulmonary microvascular endothelial cells[23] via EP4 receptor. Taken together, PGE_2 may exploit distinct EP subtypes in cell type–specific or function-dependent manners.

In order to investigate the potential participation of PGE_2 during immune inflammatory responses, its effect on IL-1β-triggered COX-2 expression in FDC-like cells was examined. PGE_2 displayed a synergistic impact and markedly augmented COX-2 levels. Since IL-1β is a pro-inflammatory cytokine released at the initial stage of inflammation,[24] our results implies that PGE_2 may play an enhancing role by inducing more prostaglandins into the immune inflammatory microenvironment. This speculation is in line with the previous suggestion of PGE_2 as an important lipid mediator of inflammation. Endogenous PGE_2 augmented pro-inflammatory

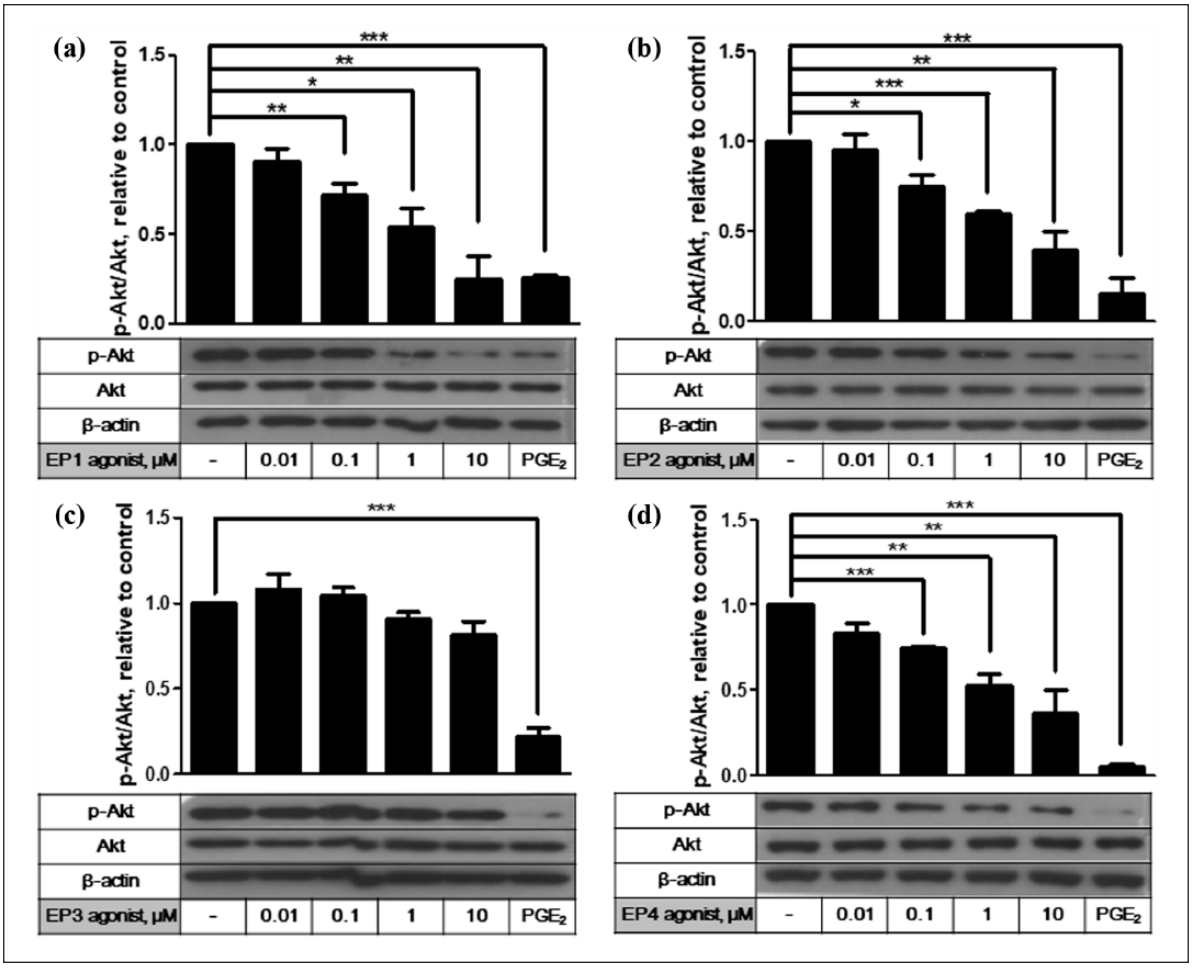

Figure 4. EP1, EP2, and EP4 agonists repress Akt phosphorylation in FDC-like cells. FDC-like cells (3×10^5 cells) were stimulated with the indicated concentrations of agonists specific to (a) EP1, (b) EP2, (c) EP3, and (d) EP4 for 30 min. PGE_2 (10 μM) was included as a positive control of Akt downregulation. Expression levels of phosphorylated Akt (p-Akt), total Akt, and β-actin were measured by immunoblotting. The intensities of p-Akt were normalized by Akt levels. Representative immunoblots and statistically analyzed data (means ± SEM) of three independent experiments are shown. Statistical significance was determined with Student's t test by comparing the values with control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

cytokine release from synovial fibroblasts[11] and monocytes.[25] The stimulating effect on COX-2 expression was also displayed by other prostaglandins, beraprost and $PGF_2\alpha$. These molecules induced COX-2 expression when added alone to FDC-like cells, which was further enhanced in the combined addition with IL-1β. Furthermore, we observed the enhancing effect with 6-keto-$PGF_1\alpha$ and thromboxane B_2 in the combined addition with IL-1β. Since 6-keto-$PGF_1\alpha$ and thromboxane B_2 do not stimulate COX-2 expression when added alone without IL-1β,[12] different from PGE_2, beraprost, and $PGF_2\alpha$, our observation implies the potential nature of prostanoids on COX-2 expression in FDC-like cells. We speculate that prostanoids with a weak inflammation-stimulating activity can be a potent trigger of COX-2 expression under a sensitized condition as demonstrated in this study. In this context, the augmenting result of another eicosanoid, hydroxyeicosatetraenoic acid, that is produced in the lipoxygenase pathway not in the COX pathway from the common substrate arachidonic acid, on COX-2 expression[26] is supportive of our suggestion. It would be interesting to examine whether weak prostanoids display such a strong enhancing activity when combined with other inflammatory stimuli than IL-1β.

In conclusion, we demonstrate in this study that PGE_2 stimulates COX-2 protein expression via EP2 and EP4 receptors and through downregulation of Akt phosphorylation and acts in synergy with IL-1β in FDC-like cells. These results imply the crucial role of the inflammatory milieu, where

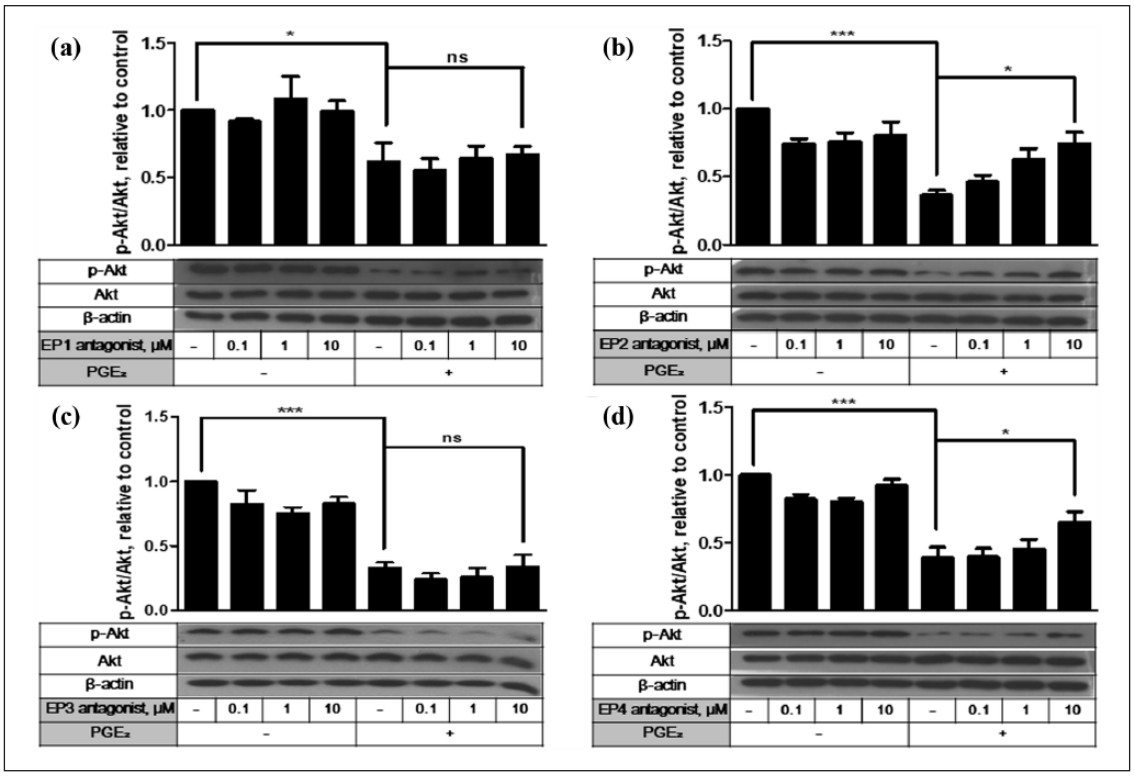

Figure 5. EP2 and EP4 antagonists impede PGE_2-induced downregulation of Akt phosphorylation. FDC-like cells (3×10^5 cells) were pre-treated with the indicated concentrations of antagonists specific to (a) EP1, (b) EP2, (c) EP3, and (d) EP4 as described in *Methods*, 1 h before the addition of PGE_2 (1 μM). After 30 min incubation, expression levels of phosphorylated Akt (p-Akt), total Akt, and β-actin were measured by the immunoblotting technique. The graphs show means ± SEM of three independent experiments with the representative immunoblots. One-way ANOVA with Tukey's multiple comparison test reveals the overall significant difference, and the intergroup significance levels are noted in the figure (*$P < 0.05$; ***$P < 0.001$; ns, non-significant).

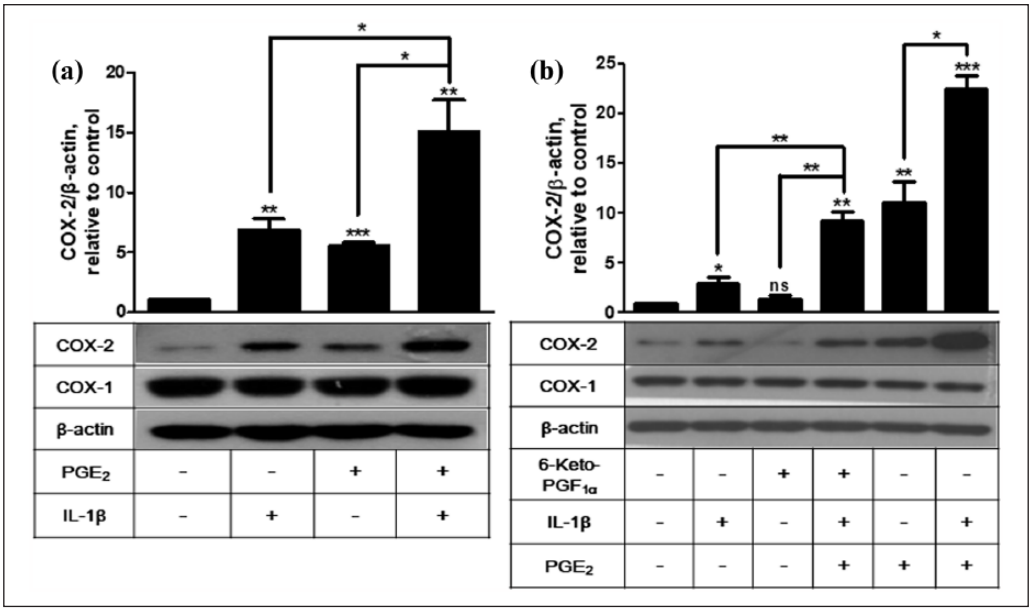

Figure 6. PGE_2 enhances IL-1β-stimulated COX-2 induction in FDC-like cells. FDC-like cells (3×10^5 cells) were cultured in presence or absence of (a) PGE_2 (10 μM) and IL-1β (1 pg/mL) or (b) 6-keto-$PGF_1\alpha$ (10 μM), IL-1β, and PGE_2 for 4 h. The expression levels of COX-1, COX-2, and β-actin in the cultured cells were measured by the immunoblotting technique. The graphs show means ± SEM of three independent experiments with the representative immunoblots. One-way ANOVA with Tukey's multiple comparison test reveals the overall significant difference, and the intergroup significance levels are noted in the figure (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, non-significant).

the combination of pro-inflammatory cytokines and non-protein modulators such as PGE_2 can lead to a potent amplification of inflammation.

Acknowledgements

J.N. and B.K. contributed equally to this work.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This research was supported by the Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science, and Technology (2016R1D1A3B03931546) and also by 2017 research grant from Kangwon National University (no. 520170426).

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