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Original Article

Prostaglandin E_2 receptors differentially regulate the output of proinflammatory cytokines in myometrial cells from term pregnant women

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Abstract: Prostaglandin (PG) E_2 plays critical roles during pregnancy and parturition. Emerging evidence indicates that human labour is an inflammatory event. We sought to investigate the effect of PGE_2 on the output of proinflammatory cytokines in cultured human uterine smooth muscle cells (HUSMCs) from term pregnant women and elucidate the role of subtypes of PGE_2 receptors (EP_1, EP_2, EP_3 and EP_4). After drug treatment and/or transfection of each receptor siRNA, the concentrations of inflammatory secreting factors in HUSMCs culture medium were detected by the corresponding ELISA kits. The results showed that, PGE_2 increased interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) output, decreased chemokine (c-x-c motif) ligand 8 (CXCL8) output in a dose-dependent manner, but had no effect on IL-1β and chemokine (c-c motif) ligand 2 (CCL-2) secretion of HUSMCs. EP_1/EP_3 agonist 17-phenyl-trinor-PGE_2 stimulated IL-6 and TNFα whilst suppressing IL-1β and CXCL8 output. The effects of 17-phenyl-trinor-PGE_2 on IL-1β and CXCL8 secretion were remained whereas its effect on IL-6 and TNFα output did not occur in the cells with EP_3 knockdown. The stimulatory effects of 17-phenyl-trinor-PGE_2 on IL-6 and TNFα were remained whereas the inhibitory effects of 17-phenyl-trinor-PGE_2 on IL-1β secretion was blocked in the cells with EP_1 knockdown. Either of EP_2 and EP_4 agonists stimulated IL-1β and TNFα output, which was reversed by EP_2 and EP_4 siRNA, respectively. The inhibitors of phospholipase C (PLC) and protein kinase C (PKC) blocked EP_1/EP_3 modulation of TNFα and CXCL8 output. PI3K inhibitor LY294002 and P38 inhibitor SB202190 blocked 17-phenyl-trinor-PGE_2-induced IL-1β and IL-6 output, respectively. The inhibitors of adenylyl cyclase and PKA prevented EP_2 and EP_4 stimulation of IL-1β and TNFα output, whereas PLC and PKC inhibitors blocked EP_2- and EP_4-induced TNFα output but not IL-1β output. Our data suggest that PGE_2 receptors exhibit different effects on the output of various cytokines in myometrium, which can subtly modulate the inflammatory microenvironment in myometrium during pregnancy.

Key words: myometrium; prostaglandin E_2; proinflammatory cytokines; pregnancy

前列腺素E_2不同受体对足月妊娠子宫肌细胞分泌炎性细胞因子的不同调节作用

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摘 要：前列腺素E_2 (prostaglandin E_2, PGE_2)在妊娠维持和分娩启动中起着关键作用。愈来愈多的研究表明，人类妊娠的维持和分娩启动是一个炎症过程。本研究用原代培养的足月妊娠人子宫平滑肌细胞(human uterine smooth muscle cells, HUSMCs)

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作为研究对象，观察PGE_2四种受体亚型(EP_1、EP_2、EP_3和EP_4)对妊娠HUSMCs分泌炎性细胞因子的影响。用药物处理和/或转染各受体siRNA后，使用ELISA试剂盒检测HUSMCs培养液中炎性分泌因子的浓度。结果显示，PGE_2可剂量依赖性促进白细胞介素6 (interleukin 6, IL-6)和肿瘤坏死因子α (tumor necrosis factor α, TNFα)分泌，而抑制趋化因子(c-x-c基序)配体8 (CXCL8)释放，但对IL-1β和趋化因子(c-c基序)配体2 (CCL-2)的分泌没有影响。EP_1/EP_3激动剂17-苯基-trinor-PGE_2刺激IL-6和TNFα的分泌，抑制IL-1β和CXCL8的分泌。转染EP_3 siRNA能逆转17-苯基-trinor-PGE_2对IL-6和TNFα分泌的影响，但是17-苯基-trinor-PGE_2对IL-1β和CXCL8的作用仍然存在。敲低EP_1可阻断17-苯基-trinor-PGE_2对IL-1β分泌的抑制效应，而17-苯基-trinor-PGE_2促进IL-6和TNFα分泌的作用仍然存在。EP_2和EP_4激动剂均可刺激IL-1β和TNFα分泌，这种效应可分别被EP_2 siRNA和EP_4 siRNA所逆转。磷脂酶C (phospholipase C, PLC)和蛋白激酶C (protein kinase C, PKC)的抑制剂可阻断17-苯基-trinor-PGE_2对TNFα和CXCL8分泌的影响。PI3K抑制剂LY294002和P38抑制剂SB202190可分别阻断17-苯基-trinor-PGE_2诱导的IL-1β和IL-6分泌。腺苷酸环化酶和PKA的抑制剂均能逆转EP_2和EP_4激动剂促进IL-1β和TNFα分泌的作用，而PLC和PKC抑制剂则可阻断EP_2和EP_4诱导的TNFα分泌，但不能阻断IL-1β的分泌。以上结果表明，PGE_2不同受体对子宫肌细胞分泌炎性细胞因子具有不同作用，提示PGE_2可能通过不同受体的精细调节来调控妊娠期子宫肌层中的炎性微环境。

关键词：子宫肌层；前列腺素E_2；促炎细胞因子；妊娠
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Preterm birth is the major cause of perinatal mortality and morbidity: 70%–80% of deaths of infants without congenital anomalies. However, despite intense laboratory and clinical investigation, the frequency of preterm birth has increased by over 30% in the last 20 years [1]. The major reason for this is that we do not understand the fundamental mechanisms governing the process of human labour.

Prostaglandins (PGs) are strongly implicated to play a central and essential role in the biochemistry of labour. Labour is associated with increased synthesis of PGs by intrauterine tissues including placenta, fetal membranes and myometrium in humans [2, 3]. PGs have been postulated to be involved in all physiologies of parturition including ripening of the cervix, membrane rupture and induction of uterine contraction [2, 3]. These properties have been exploited therapeutically to induce labour using PGE analogues [4]. However, despite widespread use, the induction of labour with PGs can be unsuccessful with a significant proportion of women requiring a caesarean section because of the failure of the induction process [5, 6]. These observations reflect, at least in part, the fact that our knowledge about the roles of PGs in pregnancy and labour is still limited.

PGs are synthesized from the precursor arachidonic acid via PG G/H synthase (PGHS) or cyclooxygenase (COX) and then via specific synthase enzymes, for example, PG E synthase (PGES) for PGE_2 and PG F synthase for PGF_2 [7]. PGE_2 is a major PG product of intrauterine tissues during pregnancy [3]. The PGE_2 receptor superfamily is composed of four distinct subtypes, designated EP_1, EP_2, EP_3 and EP_4 [8, 9]. EP_2 and EP_4 stimulate adenylyl cyclase (AC) and cAMP production, thereby resulting in muscle relaxation, whereas EP_1 and EP_3 increase phosphoinositol turnover and calcium mobilization or decrease AC and cAMP levels, leading to contraction of myometrium [8, 9].

Emerging evidence suggests that human labour is an inflammatory process, with increased leukocyte infiltration into uterine tissues and increased expression and release of numerous cytokines, including interleukin 6 (IL-6), IL-1β, chemokine (c-x-c motif) ligand 8 (CXCL8) and chemokine (c-c motif) ligand 2 (CCL-2) [10]. These cytokines promote the recruitment of leukocytes into uterine tissues, further driving the inflammatory process. Such uterine inflammatory cascades result in up-regulation of uterine activation proteins (UAPs), thereby leading to the onset of parturition [11, 12]. Classically, PGs serve as inflammatory mediators in various tissues. However, there is now increasing evidence that PGs are also involved in the resolution of inflammation and can in fact have anti-inflammatory effects [13, 14]. In the case of PGE_2, both anti-inflammatory and proinflammatory effects of PGE_2 have been demonstrated in human myometrium. Slater's group showed that PGE_2 represses IL-1β-induced inflammatory mediator output in cultured human myometrial cells from pregnant women [15, 16]. In contrast, Kandola *et al.* [17] proposed that EP_2 has proinflammatory effect since EP_2 agonist activates the proinflammatory protein COX-2 in human myometrial cells from pregnant women. These studies implicate that PGE_2 induces the complex network of inflammatory mediators in myometrium during pregnancy.

To better understand the 'inflammatory microenvironment' recruited by PGE_2 within the uterus, we used

cultured human uterine smooth muscle cells (HUSMCs) model to examine the effects of PGE_2 on the output of cytokines in pregnant human myometrium through distinct EP receptor subtype(s) and define the signaling pathways involved in different EP receptor subtypes' regulation of cytokines.

1 MATERIALS AND METHODS

1.1 *Reagents*

PGE_2, 17-phenyl-trinor-PGE_2, butaprost, U73122, chelerythrine, PD98059, LY294002, SB202190, SQ22532 and H89 were purchased from Sigma-Aldrich (St. Louis, MO, USA). TCS-2510 was provided by Tocris Bioscience.

1.2 *Isolation and culture of HUSMCs*

This study was approved by the specialty committee on ethics of biomedicine research, Second Military Medical University, Shanghai, China. Written informed consents were obtained from all the patients involved in this study.

Pregnant human myometrium tissues were obtained from patients who underwent elective cesarean section at term (37–42 weeks) in Changhai Hospital, Shanghai. HUSMCs were isolated by enzymatic dispersion as described previously [18]. Briefly, myometrial biopsies were minced and then incubated with phenol-red-free DMEM containing 1 mg/mL collagenase type II (Invitrogen, Grand Island, NY, USA), and 1 mg/mL deoxyribonuclease I (Invitrogen) at 37 °C for 45 min. The cell suspension was harvested after centrifugation and resuspended in DMEM containing 10% fetal calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 mg/mL). The cells were then plated into 25-cm^2 flasks and kept at 37 °C in 5% CO_2-95% air humidified atmosphere until confluent (2 weeks). The purity of the cultured HUSMCs were determined by immunofluorescence analysis using the antibodies which recognize muscle marker α smooth muscle actin and calponin, respectively. The results showed that more than 95% of cultured HUSMCs were α smooth muscle actin and calponin positive (supplement Fig. 1, 2, see http://www.actaps.com.cn/supplement/215Sfig.pdf).

Since *in vitro* characteristics of HUSMCs are maintained to at least 10 passages as described previously [19], all the experiments were performed with the cells at passage 2. The cells at passage 2 were placed into 6-well plates with DMEM containing 10% FCS. Following growth to ~80% confluence, the cells were changed to DMEM without FCS but containing various concentrations of PGE_2, 17-phenyl-trinor-PGE_2, butaprost or TCS-2510 in the presence or absence of the inhibitors of various kinases including U73122, chelerythrine, PD98059, LY294002, SB202190, SQ22532 or H89, then incubated for 24 h. The vehicle control was treated with the same volume of solvent (ethanol, ≤ 0.1% *v*/*v*). The supernatant and cells were harvested and stored at −80 °C.

1.3 *RNA interference*

In order to knockdown of EP_1, EP_2, EP_3 and EP_4, sequence-specific small interfering RNA (siRNA, GenePharma, Shanghai, China) was used. The sequences of EP_1, EP_2, EP_3 and EP_4 siRNA were shown in Table 1. The following nonsense siRNA (sense 5'-GAAUCUGGGAUGUUAACCATT-3'; antisense 5'-UGGUUAACAUCCCAGAUUCTG-3') was used as the negative control. The HUSMCs were transfected with siRNA targeting EP_1, EP_2, EP_3 and EP_4, or control siRNA using Lipofectamine™ RNAiMAX (Invitrogen) for 6 h, then incubated with DMEM for 18 h. The cells were incubated with 17-phenyl-trinor-PGE_2, butaprost or TCS-2510 for 24 h.

1.4 *Enzyme-linked immunosorbent assay (ELISA)*

IL-6, CCL-2, CXCL8, IL-1β and TNFα concentration in culture media of HUSMCs were measured by specific ELISA (Westang Biotech Co. Ltd., Shanghai, China) following the manufacturer's instructions.

1.5 *Western blotting analysis*

The cells were harvested in the presence of M-Per lysis buffer (Pierce Biotechnology). 50–70 μg of proteins were denatured and separated by SDS (10%)-polyacrylamide gel electrophoresis and subsequently trans-

Table 1. Sequences of EP_1, EP_2, EP_3 and EP_4 siRNA

Subtypes	Sense	Antisense
EP_1	5'-GCCAGCUUGUCGGUAUCAUTT-3'	5'-AUGAUACCGACAAGCUGGCTT-3'
EP_2	5'-GCUUUCGCCAUGACCUUCUTT-3'	5'-AGAAGGUCAUGGCGAAAGCTT-3'
EP_3	5'-GAGCGACCAUUUGGAAAGATT-3'	5'-UCUUUCCAAAUGGUCGCUCTT-3'
EP_4	5'-CUGAGGACUUUGCGAAUAUTT-3'	5'-AUAUUCGCAAAGUCCUCAGTT-3'

ferred to nitrocellulose membranes. The membranes were incubated with specific antibodies against EP_1, EP_2, EP_4 and EP_3 (1:500 dilution, Cat. No. sc-20674, sc-20675, sc-20676, and sc-55596; Santa Cruz) overnight at 4 °C. Membranes were then incubated with a secondary horseradish peroxidase-conjugated antibody and immunoreactive proteins visualized using enhanced chemiluminescence (Santa Cruz). The intensities of light-emitting bands were detected and quantified using Sygene Bio Image system (Synoptics Ltd., UK).

1.6 *Statistical analysis*

The results for all protein determinations were presented as the mean ± SEM. All data were tested for homogeneity of variance by Bartlett's test before analyzing the significance. Individual comparisons were made by one-way ANOVA (SPSS 20.0; IBM) followed by LSD test for the data which were normally distributed. Significance was achieved at $P \leq 0.05$.

2 RESULTS

2.1 The effects of PGE_2 on cytokine and chemokine output in HUSMCs

As IL-1β, IL-6, TNFα, CXCL8 and CCL-2 have been implicated in labour initiation [10, 11, 20, 21], we examined whether PGE_2 had an impact on the output of these cytokines in HUSMCs. As shown in Fig. 1, treatment of these cells with increasing concentration of PGE_2 (10^{-11}–10^{-7} mol/L) resulted in increase in IL-6 and TNFα output whilst decrease in CXCL8 output in a dose-dependent manner. PGE_2 did not have a significant effect on IL-1β and CCL-2 secretion.

2.2 EP_1 and EP_3 stimulate IL-6 and TNFα whilst suppressing IL-1β and CXCL8 output

17-phenyl-trinor-PGE_2 is an agonist of EP_1/EP_3, but has more potent activation on EP_1 [22]. Treatment of the cells with 17-phenyl-trinor-PGE_2 (10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6} mol/L) caused increase in IL-6 and TNFα output and decrease in IL-1β and CXCL8 output in a dose-dependent manner (Fig. 2*A–D*). CCL-2 output was not significantly changed upon 17-phenyl-trinor-PGE_2 treatment (Fig. 2*E*).

To further separately define the role of EP_1 and EP_3, siRNA approach was conducted to knockdown EP_1 and EP_3, respectively. Transfection of cells with EP_3 siRNA resulted in about 70% reduction of EP_3 expression (Fig. 3*A*). In the HUSMCs with EP_3 knockdown but EP_1 remained, the inhibitory effect of 17-phenyl-trinor-PGE_2 on IL-1β (Fig. 3*B*) and CXCL8 (Fig. 3*E*) output was retained, whereas the effects of 17-phenyl-trinor-PGE_2 on IL-6 (Fig. 3*C*) and TNFα (Fig. 3*D*) were lost. These data at least suggest that activation of EP_1 suppresses IL-1β and CXCL8 secretion and may also imply that EP_3 activation can influence IL-6 and TNFα secretion.

Transfection of cells with EP_1 siRNA resulted in 50% reduction of EP_1 expression (Fig. 4*A*). In these cells with EP_1 knockdown whereas EP_3 remained, the effect

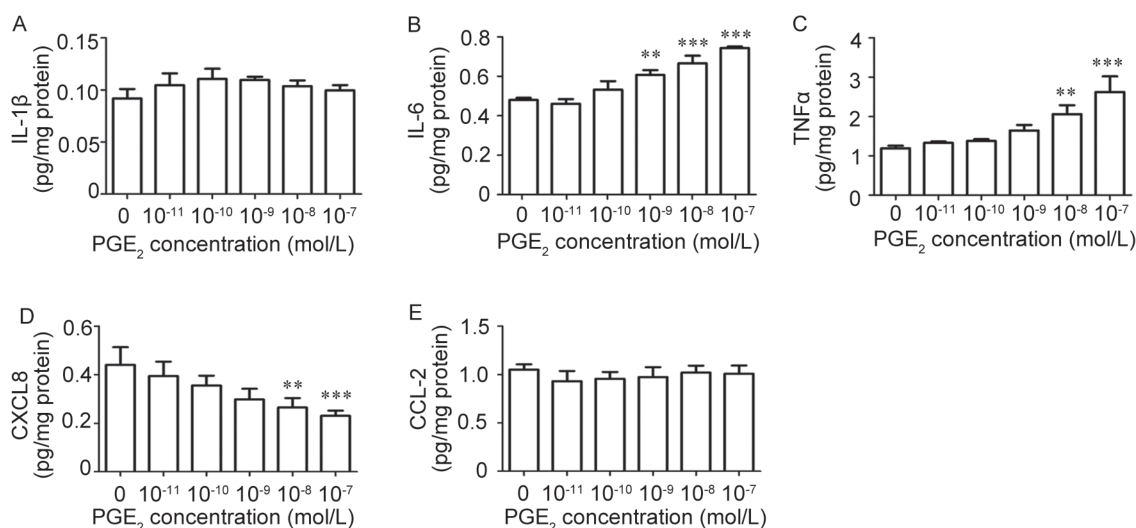

Fig. 1. The effect of prostaglandin E_2 (PGE_2) on the outputs of interleukin 1β (IL-1β), IL-6, tumor necrosis factor α (TNFα), CXCL8 and CCL-2 in human uterine smooth muscle cells (HUSMCs). HUSMCs were treated with increasing doses of PGE_2 (10^{-11} to 10^{-7} mol/L) for 24 h. The supernatants were collected for ELISA assay to determine the concentrations of IL-1β (*A*), IL-6 (*B*), TNFα (*C*), CXCL8 (*D*) and CCL-2 (*E*). Values are presented as mean ± SEM. $n = 4$. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle control.

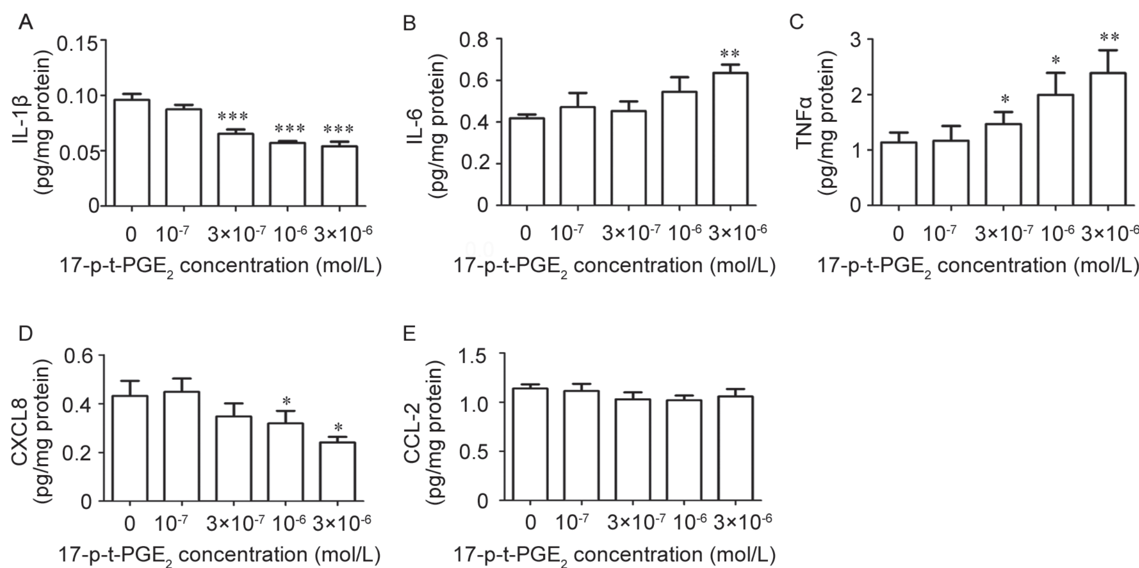

Fig. 2. The effect of 17-phenyl-trinor-PGE_2 (17-p-t-PGE_2) on the outputs of IL-1β, IL-6, TNFα, CXCL8 and CCL-2 in human uterine smooth muscle cells (HUSMCs). HUSMCs were treated with increasing concentrations of 17-phenyl-trinor-PGE_2 for 24 h. The supernatants were collected for ELISA to determine the concentrations of IL-1β (*A*), IL-6 (*B*), TNFα (*C*), CXCL8 (*D*) and CCL-2 (*E*). Values are presented as mean ± SEM. *n* = 4. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle control.

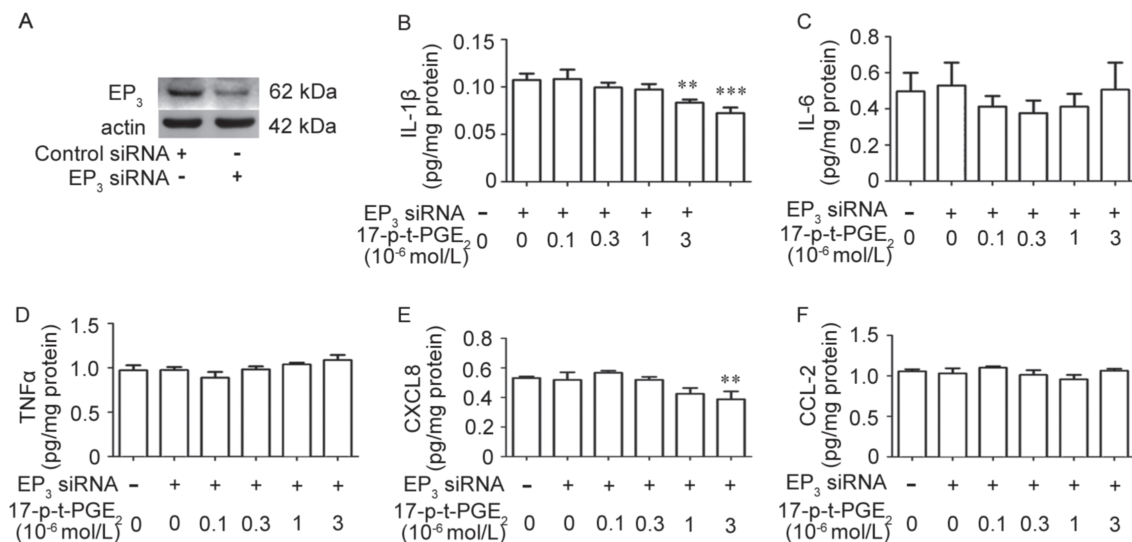

Fig. 3. The effect of 17-phenyl-trinor-PGE_2 (17-p-t-PGE_2) on the outputs of interleukin 1β (IL-1β), IL-6, tumor necrosis factor α (TNFα), CXCL8 and CCL-2 in EP_3 knockdown cells. Human uterine smooth muscle cells (HUSMCs) were transfected with specific siRNA targeting EP_3 and then treated with 17-phenyl-trinor-PGE_2 for 24 h. Supernatants were collected to determine IL-1β (*B*), IL-6 (*C*), TNFα (*D*), CXCL8 (*E*) and CCL-2 (*F*) contents. *A*: The representative bands for protein expression of EP_3 in cells transfected with EP_3 siRNA. Values are presented as mean ± SEM. *n* = 5. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with negative control.

of 17-phenyl-trinor-PGE_2 on IL-6 (Fig. 4*C*) and TNFα (Fig. 4*D*) was retained, whereas the effect on IL-1β was lost (Fig. 4*B*). These data confirm that EP_1 activation suppresses IL-1β secretion and stimulates IL-6 and TNFα secretion. In these cells, however, 17-phenyl-trinor-PGE_2 at high concentration could increase CXCL8 expression (Fig. 4*E*).

Of note, 17-phenyl-trinor-PGE_2 did not influence CCL-2 output in the cells transfected with either EP_1 siRNA (Fig. 4*F*) or EP_3 siRNA (Fig. 3*F*), suggesting that neither EP_1 nor EP_3 has an effect on CCL-2 secretion.

In addition, we also examined the effects of PGE_2

(10^{-7} mol/L) on the output of the above cytokines in the cells with EP_1 or EP_3 knockdown. As shown in Fig. 5, PGE_2 treatment still enhanced IL-6 secretion in the cells with EP_1 knockdown. In the cells of EP_3 knockdown, however, PGE_2 treatment did not affect the output of the above cytokines.

2.3 Divergent signaling pathways are involved in EP_1/EP_3 modulating output of cytokines

It is known that EP_1 and EP_3 have some common signaling pathways. For instance, EP_1/EP_3 can couple

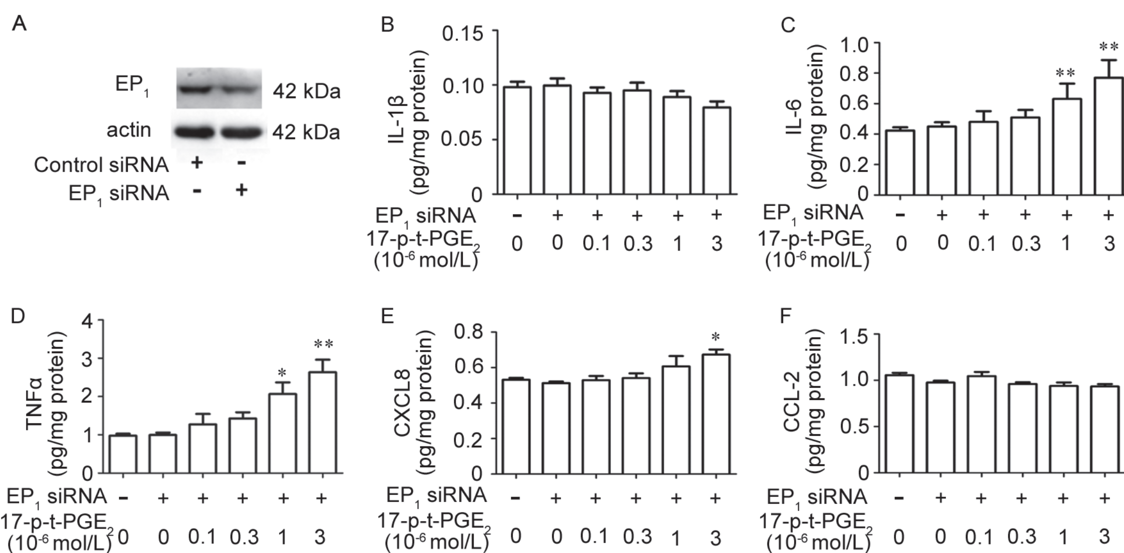

Fig. 4. The effect of 17-phenyl-trinor-PGE_2 (17-p-t-PGE_2) on the outputs of interleukin 1β (IL-1β), IL-6, tumor necrosis factor α (TNFα), CXCL8 and CCL-2 in EP_1 knockdown cells. Human uterine smooth muscle cells (HUSMCs) were transfected with specific EP_1 siRNA and then treated with 17-phenyl-trinor-PGE_2 as indicated. Following a 24 h incubation supernatants were collected to determine IL-1β (*B*), IL-6 (*C*), TNFα (*D*), CXCL8 (*E*) and CCL-2 (*F*) contents. *A*: The representative bands for protein expression of EP_1 in cells transfected with EP_1 siRNA. Values are presented as mean ± SEM. $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with negative control.

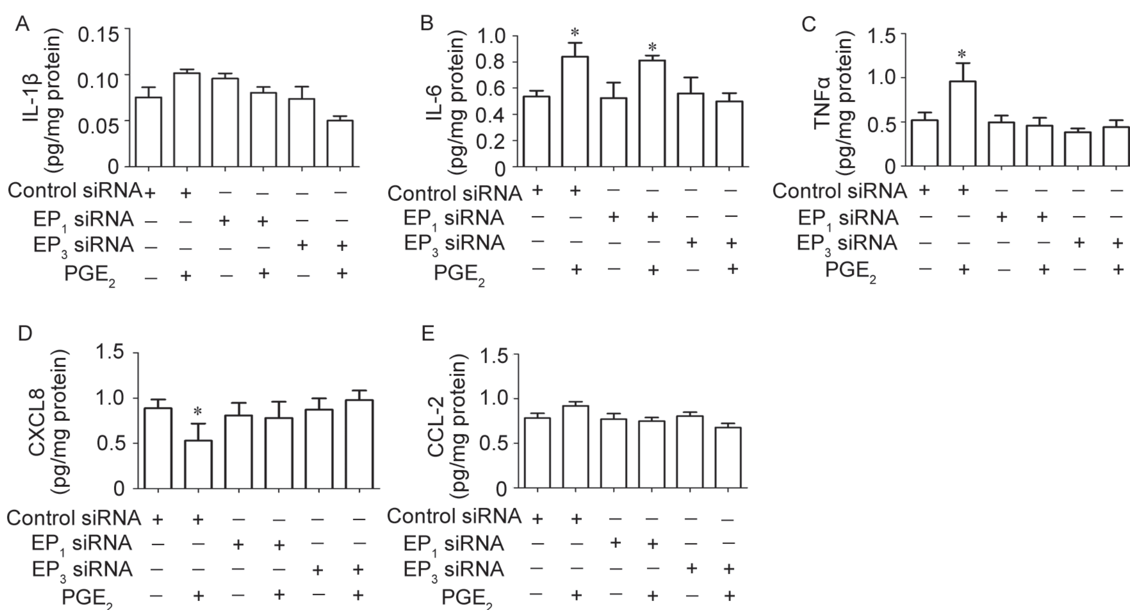

Fig. 5. The effect of PGE_2 on the output of interleukin 1β (IL-1β), IL-6, tumor necrosis factor α (TNFα), CXCL8 and CCL-2 in EP_1 or EP_3 knockdown cells. Human uterine smooth muscle cells (HUSMCs) were transfected with specific EP_1 or EP_3 siRNA and then treated with PGE_2 (10^{-7} mol/L). Following 24 h of incubation, the supernatants were collected to determine IL-1β (*A*), IL-6 (*B*), TNFα (*C*), CXCL8 (*D*) and CCL-2 (*E*) output. Values are presented as mean ± SEM. $n = 5$. $^{*}P < 0.05$ *vs* negative control.

to Gq protein and subsequently activates the effector phospholipase C (PLC) b which catalyzes the hydrolysis of membrane phosphoinositol lipids, leading to the release of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) and then activation of PKC signaling pathway [11, 12]. We therefore examined the role of PLC/PKC signaling pathway in the modulation of the cytokines by 17-phenyl-trinor-PGE_2. As shown in Fig. 6, U73122 (10^{-5} mol/L), an inhibitor of PLC, as well as PKC inhibitor chelerythrine (10^{-5} mol/L) blocked the effects of 17-phenyl-trinor-PGE_2 on TNFα (Fig. 6*C*) and CXCL8 (Fig. 6*D*) output whereas did not affect 17-phenyl-trinor-PGE_2 modulation of IL-1β (Fig. 6*A*) and IL-6 (Fig. 6*B*) output. PI3K, ERK1/2 and P38 signaling pathways can also be activated by EP subtypes [8, 9, 23–27]. LY294002 (10^{-5} mol/L), an inhibitor of PI3K, reversed 17-phenyl-trinor-PGE_2 modulation of IL-1β (Fig. 6*A*) and CXCL8 (Fig. 6*D*). PD98056 (10^{-5} mol/L), an inhibitor of ERK, totally blocked 17-phenyl- trinor-PGE_2 inhibition of TNFα and partly reversed 17-phenyl-trinor-PGE_2 modulation of IL-1β (Fig. 6*A*) and CXCL8 (Fig. 6*D*). SB202190 (10^{-5} mol/L), an inhibitor of P38, reversed the effects of 17-phenyl-trinor-PGE_2 on IL-6, TNFα and CXCL8 (Fig. 6).

2.4 EP_2 and EP_4 agonist stimulate IL-1β and TNFα output in HUSMCs

As shown in Fig. 7*A–E*, EP_2 agonist butaprost (10^{-9}–10^{-6} mol/L) stimulated IL-1β and TNFα output in a dose-dependent manner, but did not affect output of IL-6, CXCL8 and CCL-2. Transfection of EP_2 siRNA led to

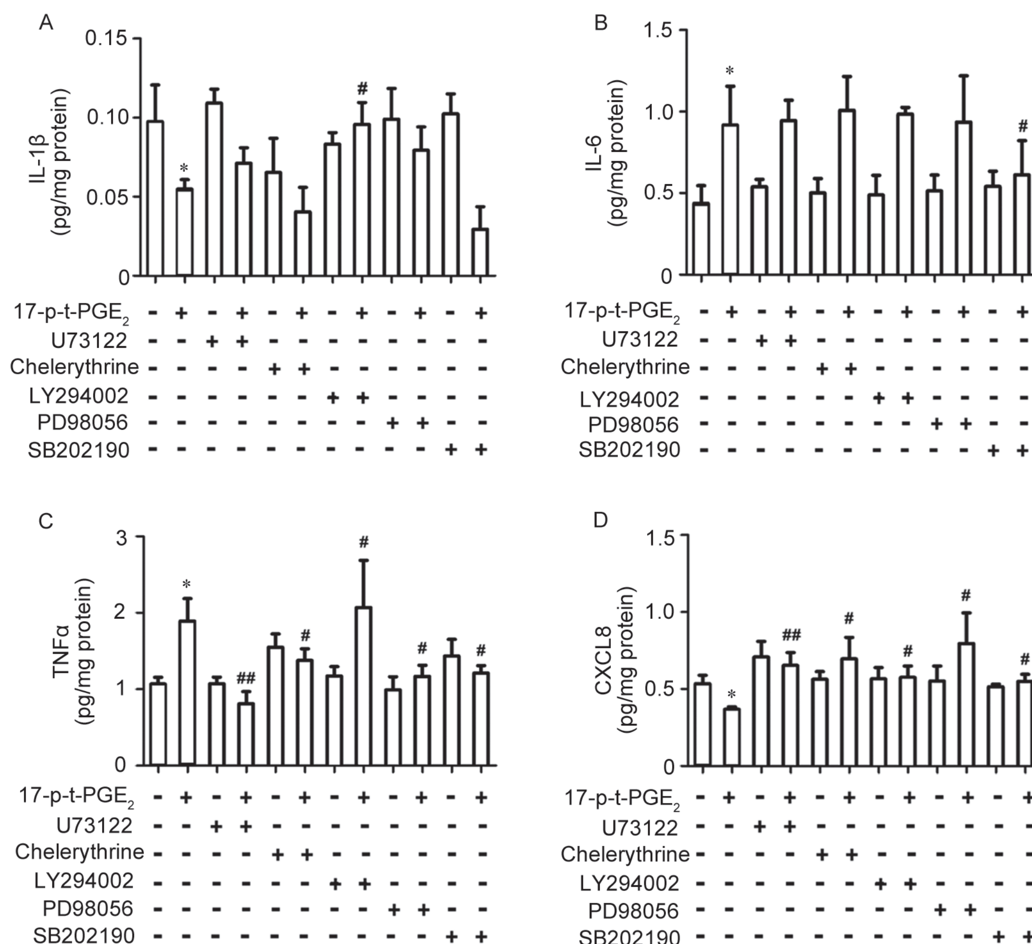

Fig. 6. The signaling pathways responsible for 17-phenyl-trinor-PGE_2 (17-p-t-PGE_2) modulation of interleukin 1β (IL-1β), IL-6, tumor necrosis factor α (TNFα) and CXCL8 output. Human uterine smooth muscle cells (HUSMCs) were treated with 17-phenyl-trinor-PGE_2 (10^{-6} mol/L) in the presence or absence of U73122 (10^{-5} mol/L), chelerythrine (10^{-5} mol/L), LY294002 (10^{-5} mol/L), PD98056 (10^{-5} mol/L) or SB202190 (10^{-5} mol/L) for 24 h. The concentrations of IL-1β (*A*), IL-6 (*B*), TNFα (*C*) and CXCL8 (*D*) in culture media were determined by ELISA. Values are presented as mean ± SEM. *n* = 4. $^{*}P < 0.05$ compared with vehicle control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with 17-p-t-PGE_2 (10^{-6} mol/L).

70% decrease in EP_2 expression and reversed butaprost stimulation of IL-1β and TNFα output (Fig. 7*F–H*).

EP_4 agonist TCS-2510 (10^{-9}–10^{-6} mol/L) treatment caused increase in IL-1β and TNFα release in a dose-dependent manner (Fig. 7*A–E*). EP_4 siRNA resulted in 60% decrease in EP_4 expression (Fig. 7*I*). In the cells

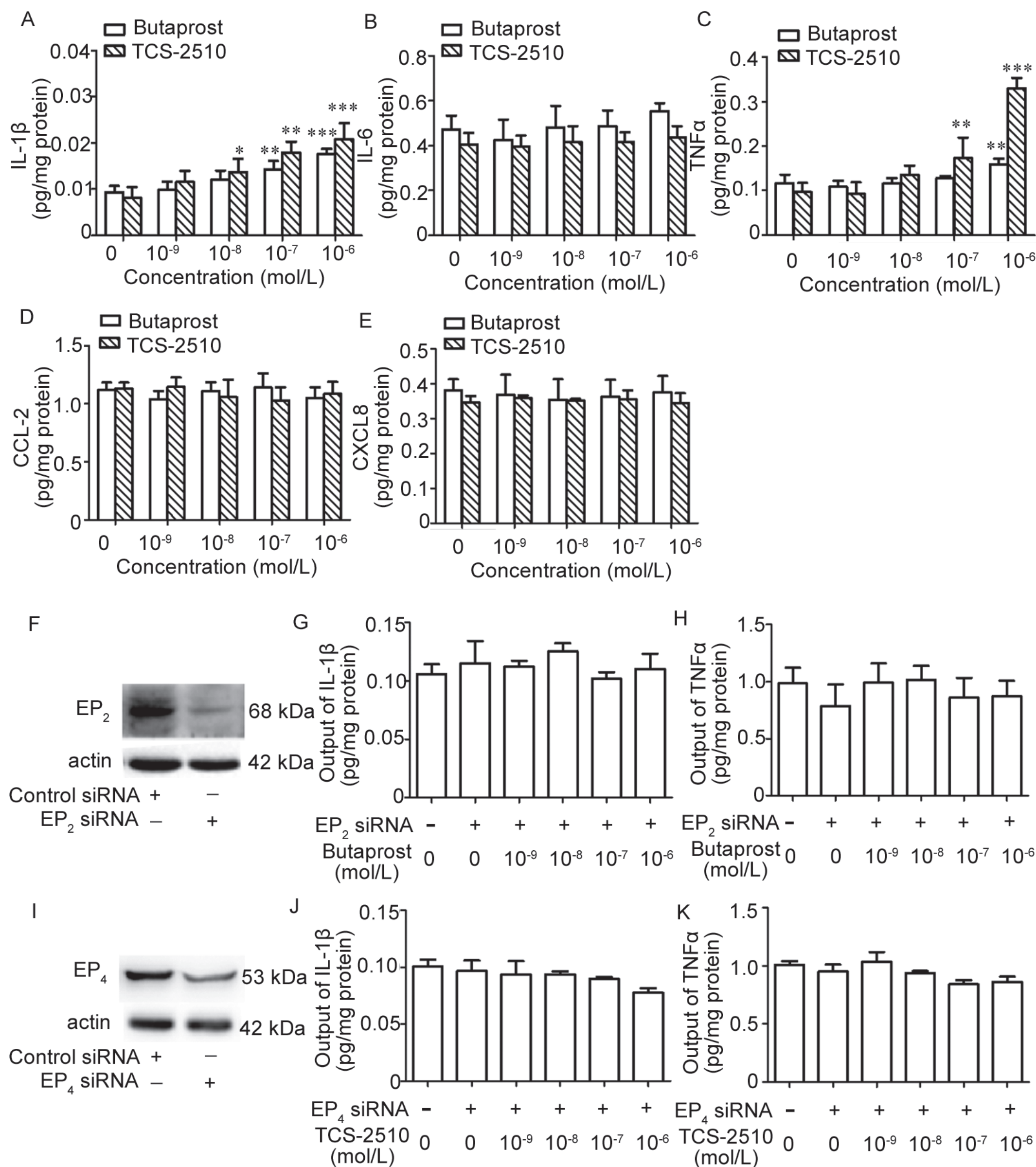

Fig. 7. The effects of EP_2 and EP_4 agonists on the outputs of interleukin 1β (IL-1β), IL-6, tumor necrosis factor α (TNFα), CXCL8 and CCL-2 in human uterine smooth muscle cells (HUSMCs). *A–E*: The effects of EP_2 and EP_4 agonists on cytokine output in myometrial cells. HUSMCs were treated with increasing concentrations of butaprost (10^{-9} to 10^{-6} mol/L) or TCS-2510 (10^{-9} to 10^{-6} mol/L) for 24 h. Culture media were then collected, and the concentrations of IL-1β (*A*), IL-6 (*B*), TNFα (*C*), CXCL8 (*E*) and CCL-2 (*D*) were determined by ELISA. *F–H*: The effect of EP_2 siRNA on butaprost-induced cytokine output. HUSMCs were transfected with specific EP_2 siRNA and then treated with butaprost for 24 h. The concentrations of IL-1β and TNFα in culture media were determined by ELISA. *F*: The representative bands for protein expression of EP_2 in cells transfected with EP_2 siRNA. *G*: The concentration of IL-1β. *H*: The concentration of TNFα. *I–K*: The effect of EP_4 siRNA on TCS-2510-induced cytokine output. HUSMCs were transfected with specific EP_4 siRNA and then treated with TCS-2510 for 24 h. *I*: The representative bands for protein expression of EP_4 in cells transfected with EP_4 siRNA. *J*: The concentration of IL-1β. *K*: The concentration of TNFα. Values are presented as mean ± SEM. $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle control.

transfected with EP_4 siRNA, TCS-2510 induction of IL-1β and TNFα output did not occur (Fig. 7*J*, *K*).

2.5 The action of EP_2 and EP_4 is through multiple signaling pathways

As mentioned, classically, EP_2 and EP_4 couple to Gs protein, and subsequently activate the effector AC which stimulates cAMP production, leading to PKA activation. More recently, Kandola *et al.* [17] have demonstrated that in addition to Gs, EP_2 can also couple to Gq protein and activate PLC and its downstream signaling. We therefore examined whether these signaling pathways are involved in EP_2 and EP_4 stimulation of IL-1β and TNFα output. As shown in Fig. 8, AC inhibitor SQ22532 (10^{-5} mol/L) and PKA inhibitor H89 (10^{-5} mol/L) totally blocked the effects of EP_2 and EP_4 on IL-1β and TNFα secretion. PLC inhibitor U73122

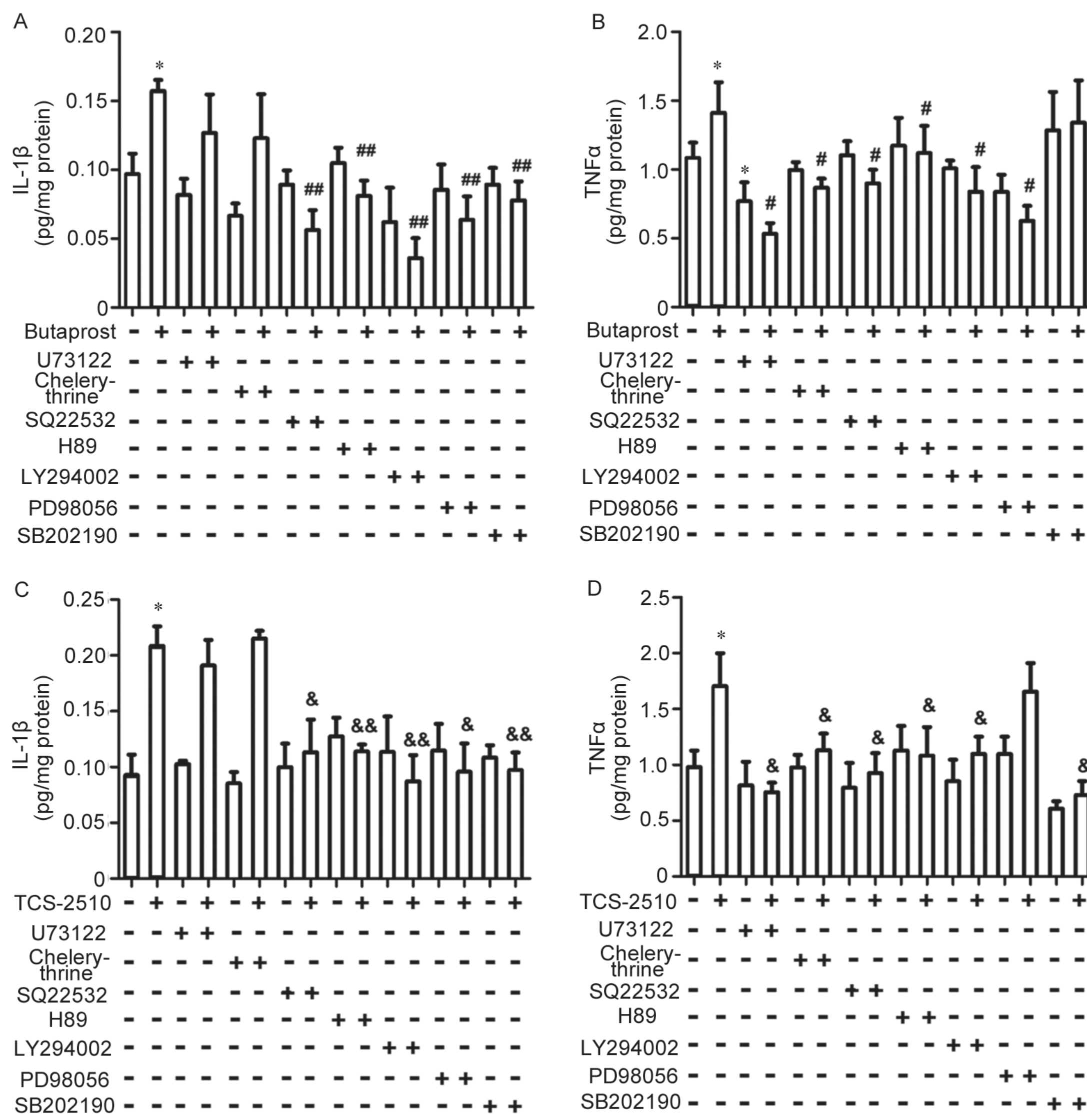

Fig. 8. The signaling pathways responsible for EP_2 and EP_4 modulation of interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα) output. *A*, *B*: Human uterine smooth muscle cells (HUSMCs) were treated with butaprost (10^{-6} mol/L) in the presence or absence of U73122 (10^{-5} mol/L), chelerythrine (10^{-5} mol/L), SQ22532 (10^{-5} mol/L), H89 (10^{-5} mol/L), LY294002 (10^{-5} mol/L), PD98056 (10^{-5} mol/L) or SB202190 (10^{-5} mol/L) for 24 h. The concentrations of IL-1β (*A*) and TNFα (*B*) in culture media were determined. *C*, *D*: HUSMCs were treated with TCS-2510 (10^{-6} mol/L) in the presence or absence of U73122 (10^{-5} mol/L), chelerythrine (10^{-5} mol/L), SQ22532 (10^{-5} mol/L), H89 (10^{-5} mol/L), LY294002 (10^{-5} mol/L), PD98056 (10^{-5} mol/L) or SB202190 (10^{-5} mol/L) for 24 h. The concentrations of IL-1β (*C*) and TNFα (*D*) in culture media were determined. Values are presented as mean ± SEM. $n = 4$. $^{*}P < 0.05$ compared with vehicle control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with butaprost (10^{-6} mol/L); $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared with TCS-2510 (10^{-6} mol/L).

(10^{-5} mol/L) and PKC inhibitor chelerythrine (10^{-5} mol/L) reversed EP_2- and EP_4-induced TNFα but not IL-1β output. LY294002, PD98056 and SB202190 prevented EP_2- and EP_4-induced IL-1β output. LY294002 and PD98056 blocked the effect of EP_2 on TNFα secretion, while LY294002 and SB202190 could reverse EP_4-induced TNFα secretion.

3 DISCUSSION

Uterine smooth muscle cells are able to produce various cytokines such as IL-1β, IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF), TNFα, IL4, CXCL8 and CCL-2 [11, 15, 16, 28], which are associated with "an immune microenvironment" in uterus during pregnancy. At the end of pregnancy, production of proinflammatory cytokines such as IL-1β and IL-6 as well as chemokines CXCL8 and CCL-2 is greatly increased [20, 28] and these factors can promote the recruitment and maturation of leukocytes in uterus, further driving the inflammatory process. Our recent study has demonstrated that corticotropin-releasing hormone (CRH), which plays a critical role in initiation of labour in humans, stimulates the production of IL-1β, IL-6, TNFα, CXCL8 and CCL-2 in pregnant human myometrial cells [28]. In the present study, we showed that basal output of IL-1β, IL-6, TNFα and CXCL8 in pregnant HUSMCs was also modulated by PGE_2 and its receptors. Given that CRH and PGs play important roles in maintenance of pregnancy and initiation of labour in humans, it suggests that regulation of cytokine production within the uterus could be one of the mechanisms of these hormones controlling pregnancy and parturition.

There are a few studies about PGE_2 regulation of inflammatory mediator production in human myometrium. The study by Slater's group demonstrated that PGE_2 represses IL-1β-induced inflammatory mediators such as CCL-2, CXCL8 and GM-CSF output in cultured HUMSCs. However, they did not show any effects of PGE_2 on the basal levels of these cytokines in pregnant myometrium [16]. In the present study, we found that PGE_2 inhibits basal CXCL8 output in pregnant HUMSCs. However, we showed that PGE_2 exhibited divergent effects on the secretion of various cytokines. Besides it suppresses CXCL8, it stimulated IL-6 and TNFα output in pregnant HUMSCs. In various cells, PGE_2 exhibits both pro- and anti-inflammatory effects. For instance, PGE_2 suppresses the production of various inflammatory mediators including TNFα, IL-6, IL-12, CCL-3, CCL-4 and CCL-10 induced by LPS in immune cells [29–31]. In contrast, some studies have demonstrated that PGE_2 stimulates CXCL8 production in some tissues including microvascular epithelia and endometrium [32, 33]. Together, it suggests that the effects of PGE_2 on basal and induced secretion of various cytokines might be different and also dependent on the context of tissues.

As mentioned, different expression pattern of EP subtypes may account for divergent effects of PGE_2 on inflammatory mediators in different tissues. A number of studies have defined the role of individual EP subtype in PGE_2 modulation of inflammatory mediators in some tissues. For instance, PGE_2 promotes IL-23 production via EP_4 but suppresses IL-23 through EP_2 in dendritic cells [34], suggesting that EP subtypes exhibit opposite effects on some cytokines. In the present study, we found that EP_1/EP_3 and EP_2/EP_4 had opposite effects on IL-1β output, while these four receptors had a consistent effect on TNFα secretion. The study by Slater's group showed that EP_2 and EP_4 mediate PGE_2 repressing IL-1β-induced CCL-2, CXCL8 and GM-CSF production in pregnant HUSMCs [16]. Here, we showed that EP_2/EP_4 did not have an inhibitory effect on basal CXCL8 output in pregnant HUSMCs, but stimulated IL-1β and TNFα secretion. Moreover, we also found that four EP subtypes had no effect on basal CCL-2 output. Taken together, it would again indicate that EP subtypes had differential effects on basal and induced production of cytokines in human myometrium.

Of note, our results showed that the inhibitory effect of EP_1/EP_3 agonist on CXCL8 output did not occur in the cells with EP_1 knockdown. Instead, CXCL8 secretion was enhanced by it at a high concentration in these cells. Interestingly, suppression of IL-1β by EP_1/EP_3 agonist was lost in the cells with EP_1 knockdown. Given that prior study has demonstrated that IL-1β induces CXCL8 secretion in pregnant HUSMCs [16], it may indicate that inhibition of 17-phenyl-trinor-PGE_2 might result from the suppression of secretion. Nevertheless, regulatory mechanism responsible for CXCL8 secretion by EP_1 and EP_3 remains to be further elucidated.

As mentioned before, EP receptors can induce multiple signaling pathways. In the present study, we found that both PLC/PKC and AC/PKA signaling pathways were involved in the EP_2 and EP_4 stimulation of TNFα secretion, while AC/PKA signaling was involved in EP_2 and EP_4 stimulation of IL-1β secretion. These data are con-

sistent with other studies that demonstrated that EP_2 or EP_4 can couple to dual G proteins, Gq and Gs, and subsequently activate downstream signaling pathways [8, 17, 34]. Classically, EP_1 and EP_3 couple to Gq protein and activate PLC/PKC signaling pathways, and PI3K, ERK and P38 can be activated by various EP receptors including EP_1 and EP_3 [8, 9, 23–27]. Using various inhibitors of the above signaling pathways, our data indicate that EP_1/EP_3 stimulation of TNFα production and suppression of CXCL8 production might be dependent on PLC/PKC and P38 signaling pathways and the effects of EP_1/EP_3 on IL-1β might be dependent on PI3K signaling pathways. However, whether the above signaling pathways are activated by four EP subtypes in pregnant HUSMCs requires to be confirmed in the subsequent studies.

During human pregnancy, the uterus undergoes remarkable changes. It is the pro-pregnancy and anti-inflammatory state during most of pregnancy, whereas at the end of pregnancy it switches to the pro-labour, proinflammatory state. Numerous factors including PGs are involved in this transition via endocrine, paracrine and autocrine pathways [2]. As mentioned before, myometrial smooth muscle cells synthesize numerous cytokines which contribute to the immune microenvironment in myometrium. Our previous studies have demonstrated that $PGF_{2α}$ stimulates IL-6, CCL-2 and CXCL8 secretion through divergent signaling pathways in pregnant HUSMCs [35]. The present study showed that four PGE_2 receptors have differential effects on the output of various cytokines including IL-1β, IL-6 and CXCL8. Interestingly, in the present study, we showed that changed expression of one EP subtype using siRNA approach would lead to an alternation in the secretion pattern of cytokines, suggesting that the effects of PGE_2 on the output of cytokines are dependent on the ratio of four EP subtypes. Interestingly, prior studies have suggested that the levels of four PGE_2 receptors are changed with pregnancy [36, 37], which indicates that the ratio of four EP subtypes in myometrium is changed during pregnancy. Thus, it might imply that PGE_2 modulates the immune microenvironment in uterus through a fine tune mechanism, which might contribute to state transformation of uterus during pregnancy.

In conclusion, our study demonstrate that 4 EP subtypes exhibit different effects on chemokine and cytokine output in myometrium which can subtly modulate the immune microenviroment in myometrium during pregnancy.

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