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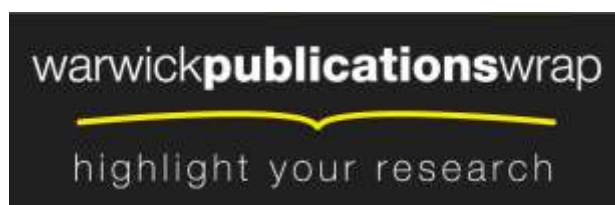
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## **Cyclic AMP increases COX-2 expression *via* mitogen-activated kinase in human myometrial cells**

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### **Abstract**

Cyclic AMP (cAMP) is the archetypal smooth muscle relaxant, mediating the effects of many hormones and drugs. However, recently PGI<sub>2</sub>, acting *via* cAMP/PKA, was found to increase contraction-associated protein expression in myometrial cells and to promote oxytocin-driven myometrial contractility. Cyclo-oxygenase-2 (COX-2) is the rate-limiting enzyme in prostaglandin synthesis, which is critical to the onset and progression of human labour. We have investigated the impact of cAMP on myometrial COX-2 expression, synthesis and activity. Three cAMP agonists (8-bromo-cAMP, forskolin and rolipram) increased COX-2 mRNA expression and further studies confirmed that this was associated with COX-2 protein synthesis and activity (increased PGE<sub>2</sub> and PGI<sub>2</sub> in culture supernatant) in primary cultures of human myometrial cells. These effects were neither reproduced by specific agonists nor inhibited by specific inhibitors of known cAMP-effectors (PKA, EPAC and AMPK). We then used shRNA to knockdown the same effectors and another recently described cAMP-effector PDZ-GEF1-2, without changing the response to cAMP. We found that MAPK activation mediated the cAMP effects on COX-2 expression and that PGE<sub>2</sub> acts through EP-2 to activate MAPK and increase COX-2. These data provide further evidence in support of a dual role for cAMP in the regulation of myometrial function.

**Keywords:** human myometrium • cyclic AMP • cyclooxygenase-2 • prostaglandins

### **Introduction**

Preterm delivery is the prime cause of perinatal mortality and morbidity [1, 2]. However, despite intense laboratory and clinical investigations, the frequency of preterm birth has increased by over 30% in the last 20 years [3]. The early onset of labour is the most frequent cause of preterm birth and is most commonly related to intrauterine inflammation, uterine overdistension and placental abruption [4]. These factors shift the balance from myometrial quiescence to contractility and promote the onset of labour.

The second messenger, cAMP, influences a wide array of physiological and pathological events including smooth muscle contractility and inflammation. Indeed, both physiological (relaxin, CRH and CGRP) and pharmacological ( $\beta_2$ -agonists) agents act *via* cAMP to induce myometrial relaxation. However, therapeutically in the management of preterm labour (PTL),  $\beta_2$ -agonists are limited both by severe, potentially life threatening side effects and tachyphylaxis, the latter mediated by  $\beta_2$ -agonist-induced down-regulation of myometrial  $\beta$ -adrenergic receptors [5, 6]. Consequently, other mechanisms to increase myometrial cAMP levels have been explored and a recent publication showed that using the phosphodiesterase type 4 inhibitor, rolipram, successfully reduced rates of PTL in a mouse model [7].

Prostaglandins (PGs) play a critical role in the onset of preterm and term labour, ripening the cervix [8] and promoting myometrial contractility [9]. These properties have been successfully exploited therapeutically on the one hand to induce labour and on

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the other by using inhibitors of PG synthesis to prevent PTL [9–11]. The key enzyme in PG synthesis is COX, and at least three isoforms are present in human myometrium, of which COX-2 is the most important. COX-2 is highly regulated by both transcriptional and post-transcriptional mechanisms [12, 13] and can be stimulated by growth factors, cytokines and endotoxins; its expression is increased in myometrium and amnion with the onset of labour [14, 15]. We have previously reported that the inflammatory cytokine, IL-1 $\beta$ , promotes COX-2 expression *via* NF- $\kappa$ B activation and that both IL-1 $\beta$  and mechanical stretch also act *via* MAPK to up-regulate COX-2 expression [16]. Previous studies have shown that cAMP is able to down-regulate both NF- $\kappa$ B and MAPK activity in a variety of tissues [17, 18] and could therefore be therapeutically useful in the prevention of PTL. However, interestingly, a recent study found that activation of the cAMP/PKA pathway by PGI<sub>2</sub> leads to increased expression of the contraction associated proteins, connexin 43,  $\alpha$ -SMA, h-caldesmon, calponin and SM2-MHC, suggesting that in response to certain signals, cAMP might promote the process of myometrial activation prior to the onset of labour [19]. Given that cAMP has the potential to be an effective tocolytic, but may also in some circumstances promote myometrial contractility, we have carried out this study to define whether cAMP increases myometrial COX-2 expression and studied the mechanisms responsible.

## Materials and methods

### Tissue collection

The local ethics committee approved the study and women donating tissue gave informed written consent. Human myometrial biopsies were taken at near term labour (approximately 39 weeks) from the upper margin of the uterine lower segment incision at the time of elective caesarean section prior to the onset of labour. The samples were processed for myocyte isolation and culture.

### Isolation and culture of human myometrial cells

The myometrial tissue was carefully dissected and washed in ice-cold PBS several times. The tissue samples were digested for about 45–60 min. at 37°C in a collagenase solution 0.5 mg/ml collagenase 1A (Sigma-Aldrich Co. Ltd., Poole, Dorset, UK), 0.5 mg/ml collagenase XI (Sigma-Aldrich Co. Ltd.), 1 mg/ml bovine serum albumin in DMEM (Sigma-Aldrich Co. Ltd.). Digestion was stopped by addition of DMEM supplemented with 7.5% foetal calf serum (FCS; Sigma-Aldrich Co. Ltd.). The myometrial tissues suspension was agitated to further disperse the cells. The resulting suspension was then passed through a cell strainer (70  $\mu$ m nylon cell strainer) and individual cells were collected by centrifugation at 3000 r.p.m. for 5 min. After washing, cells were grown in DMEM with supplementation of 7.5% FCS, 1% l-glutamine and 1% penicillin–streptomycin at 37°C and 5% CO<sub>2</sub>. The myometrial cells were used at either second or third passages. The culture medium was changed after 24 hrs and then every other day. Cells were exposed to different treatments as described for individual

experiments. Cells were serum starved overnight prior to initiation of experiments.

## Materials

In the following experiments, we use the different treatments and the final concentration of them was: forskolin 100  $\mu$ M (Sigma-Aldrich Co. Ltd.), PGE<sub>2</sub> 10  $\mu$ M, 8bromo-cAMP (Sigma-Aldrich Co. Ltd.), 250  $\mu$ M, rolipram 10  $\mu$ M (Sigma-Aldrich Co. Ltd.), Sp-6-phe-cAMP (PKA agonist 100nM; BIOLOG Life Science Institute, Bremen, Germany), 8pCPT-2'-O-Me-cAMP (Epac agonist, 50  $\mu$ M; BIOLOG Life Science Institute), AICAR (AMPK agonist, 100 $\mu$ M; Merck Chemicals Ltd., Beeston, Nottingham, UK), KT5720 (PKA inhibitor 10 $\mu$ M; Sigma-Aldrich Co. Ltd.), brefeldin A (Epac inhibitor 100  $\mu$ M; Sigma-Aldrich Co. Ltd.), compound C (AMPK inhibitor 100  $\mu$ M; Merck Chemicals Ltd.), SB203580 (p38 inhibitor 10  $\mu$ M; Tocris Cookson Ltd., Avonmouth, Bristol, UK), U0126 [ERK inhibitor 10  $\mu$ M; New England Biolabs (UK) Ltd., Hitchin, Hertfordshire, UK], SP600125 (JNK inhibitor 20  $\mu$ M; Tocris Cookson Ltd.), PKG inhibitor (Rp-8-Bromo- $\beta$ -phenyl-1, N2-ethenoguanosine 3':5'-cyclic monophosphorothioate sodium salt hydrate, 15  $\mu$ M; (Sigma-Aldrich Co. Ltd.). Butaprost (EP-2 agonist 10  $\mu$ M; Cayman Chemical Co., Ann Arbor, MI, USA), EP-4 agonist (10  $\mu$ M, a gift from GlaxoSmithKline, Brentford, Middlesex, UK).

## RT-PCR of COX-2 mRNA

Total RNA was extracted and purified from human myometrial cells grown in six-well plates using RNeasy minikits purchased from Qiagen (Catalog No. 74106; Qiagen Ltd., Crawley, West Sussex, UK). After RNA quantification, 1.0  $\mu$ g was reverse transcribed with oligo dT random primers using MuLV reverse transcriptase (Applied Biosystems Ltd., Warrington, Cheshire, UK). Primer sets for COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed and obtained from Invitrogen Ltd. (Paisley, UK; Table 1). Assays were validated for all primer sets by confirming that single amplicons of appropriate size and sequence were generated according to predictions. Quantitative PCR was performed in the presence of SYBR Green (Applied Biosystems Ltd.), and amplicon yield was monitored during cycling in a RotorGene Sequence Detector (Corbett Research Ltd., Mortlake, Sydney, Australia) that continually measures fluorescence caused by the binding of the dye to double-stranded DNA. Pre-PCR cycle was 10 min. at 95°C followed by up to 45 cycles of 95°C for 20 sec., 58–60°C for 20 sec. and 72°C for 20 sec. followed by an extension at 72°C for 15 sec. The final procedure involves a melt over the temperature range of 72–99°C rising by 1° steps with a wait for 15 sec. on the first step followed by a wait of 5 sec. for each subsequent step. The cycle in which fluorescence reached a preset threshold (cycle threshold) was used or quantitative analyses. The cycle threshold in each assay was set at a level where the exponential increase in amplicon abundance was approximately parallel between all samples. All mRNA abundance data were expressed relative to the amount of constitutively expressed GAPDH.

## Western blotting

Monolayers of human myometrial cells were lysed in cell lysis buffer obtained from New England BioLabs (UK) Ltd., scraped and collected in Eppendorf tubes. Samples were centrifuged at 13,000  $\times g$  for 15 min. at 4°C. Supernatant were then transferred and stored at –80°C. Protein

**Table 1** Primer sequence, GenBank accession numbers and size of PCR products (bp)

Gene primer sequence (5'–3')	GenBank accession no.	Size of PCR products (bp)
<i>COX-2</i>		
Forward 5'-TGTGCAACACTTGAGTGG CT-3'	AY151286	220
Reverse 5'-ACTTCTGTACTGCGGGTGG-3'		
<i>GAPDH</i>		
Forward 5'-TGATGACATCAAGAAGGTGGTGAAG-3'	BC014085	297
Reverse 5'-TCCTTGGAGGCCATGTAGCCAT-3'		

samples were denatured by heating 70°C for 10 min. and ran on a 10% SDS-PAGE for 30 min. at 80 V, and then 40–60 min. at 125 V, followed by a transfer to a Hybond ECL nitrocellulose membrane (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK). The membrane was blocked with 5% milk protein solution overnight or for 1 hr, washed and hybridized with the primary antibody overnight at 4°C in a fresh blocking buffer (1×PBS, 1% milk protein and 0.1% Tween-20). Antibodies used included COX-2, EPAC (sc-1746, sc-8880, UK; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), p38, ERK, JNK, phospho-p38, phospho-ERK, phospho-JNK, PKA, AMPK [9212, 9102, 9252, 9212, 9101, 9251, 2532; New England BioLabs (UK) Ltd.], PDZ-GEF<sub>1,2</sub> (WH0009693M1, WH0051735 M1; Sigma-Aldrich Corp., St. Louis, MO, USA) and β-actin (A1978; Sigma-Aldrich Chemie GmbH, Munich, Germany). Subsequently, the membrane was incubated with the secondary antibody at room temperature for 2 hrs. For ECL detection of horseradish peroxidase, ECL plus (GE Healthcare UK Ltd.) was used. Exposure for detection was at 25°C for 1–5 min.

### PGE<sub>2</sub>, PGI<sub>2</sub> and PGF<sub>2α</sub> Measurement

Following stimulation, the medium was collected and frozen at –80°C prior to analysis. Levels of PGE<sub>2</sub>, PGI<sub>2</sub> and PGF<sub>2α</sub> were measured by ELISA using a Luminex Prostaglandin kit [R&D Systems, Inc., Minneapolis, MN, USA, Catalog. No. KGE004B; Enzo Life Sciences (UK) Ltd., Exeter, UK; Catalog No. 900-025; Cayman Chemical Co., Catalog No. 51601]. The ELISA was performed according to the manufacturer's protocol. The concentrations of PGE<sub>2</sub>, PGI<sub>2</sub> and PGF<sub>2α</sub> are expressed as pg/10<sup>5</sup> cells.

### Transient gene transfection (knockdown PKA, Epac, AMPK, PDZ-GEF<sub>1,2</sub> and COX-2 promoter-luciferase assay)

Cells were cultured in 24-well plates to about 80% confluence and then transfected by using Gene-Juice transfection reagent (Novogen Ltd. Windsor Berkshire, UK) according to the manufacturer's protocol. A

**Table 2** ShRNA sequences used to knockdown cAMP effectors

<i>ShRNA DNA sequences</i>	
Genes	
shRNA PKA	GGAACCACTATGCCATGAAGATCCTCGAC
	GGAGATGTTCTCACACCTACGGCGGATCG
	GTCTCCATCAATGAGAAGTGTGGCAAGGA
shRNA Epac	CTGGATTGCCATCTACCAGAGGAAGGTGG
	TGTTCTGCTCTTTGAACCACACAGCAAGG
	CTGCGTGTGGACAAGCAGGACTTCAACCG
shRNA AMPK	GCTTCCTCCAGAACTCTCAGACCTGGTG
	TACTCAACATGGTGTGAGAAGGATGCAC
	AGAAGATTCGGAGCCTTGATGTGGTAGGA
shRNA PDZ-GEF <sub>1</sub>	TTGGCAGTTGCTACCATCTCATAATAGA
	GGAAGAATCCTGTGACAAGCACTACTCC
	GAGTGATTGATGCTGAGGCTCAAGGAA
shRNA PDZ-GEF <sub>2</sub>	CAATGTCAGTGAGGCGAGAAGTCTGTGCT
	TGGTCAGTCTCAAGATGACAGCATAGTAG
	ATGGACGAGGAGAGTCTTCAGACATTATC
shRNA PDZ-GEF <sub>2</sub>	GTCTGTGACTACGGAAGAAACCAAGCCTG
	ACTCATCTTGCACTTACTGTGAAGACCAA
	GGACTGAACAAGAGAAATCTGGTGTTCCT
	CTTCCAGAAGGACCTGTTGATTCTGAGGA
	AGCACGCTATGAGAGATACAGTGGCAATC

reporter construct containing 2419-bp of the promoter region of human COX-2 gene was used. The expression constructs and COX-2 reporter vector were co-transfected at concentrations of 300 ng/well and SV40-Renilla vector [pRL-SV40; Promega (UK) Ltd., Southampton, Hampshire, UK], which was used as a control for transfection efficiency at concentrations of 100 ng/well. The empty expression vector pSG5 and pGL4-Luc were included as filler constructs so that the total amount of transfected DNA per well was constant. Firstly, cells were treated for 24 hrs with transfection reagent and then with the specific stimulus for another 24 hrs. Finally, luciferase activity was measured by using a dual firefly/renilla luciferase assay [Lucite was obtained from PerkinElmer, Buckinghamshire, Seer Green, UK and Coelenterazine was from CN Biosciences (UK) Ltd., Beeston, Nottingham, UK]. All transfections were performed in triplicates. Results of luciferase activity was first normalized to the level of Renilla luciferase activity and then calculated as fold induction relative to either the expression of vehicle-treated group, or the control empty expression vector. Based on preliminary transfection experiments, after transfection of relevant ShRNAs (OriGene Technologies, Rockville, MD, USA; Table 2) for the first 24 hrs, the medium was changed to one containing 7.5% serum, then after another 24 hrs, the medium was changed to one containing

0.1% puromycin (final concentration 1 ng/ml) medium. After 24–48 hrs, further transfections were carried out with the COX-2 promoter or treat with various stimuli for another 24 hrs.

## Statistical analysis

All data were initially tested for normality using a Kolmogorov–Smirnov test. Normally distributed data were analysed using a Student's *t*-test for two groups and an ANOVA followed by a Dunnett's or Bonferroni's *post-hoc* test for three groups or more. Data that were not normally distributed were analysed using a Wilcoxon matched pair test for paired data and when comparing three groups or more a Friedman's test, with a Dunn's multiple comparisons *post-hoc* test.  $P < 0.05$  was considered statistically significant.

## Results

### Induction of COX-2 mRNA expression, COX-2 protein and PG synthesis by cAMP

We initially investigated whether cAMP increased COX-2 mRNA expression by exposing human myometrial cells to forskolin (100  $\mu$ M) and found increased COX-2 expression at 1 and 6 hrs (Fig. 1A). We confirmed these findings with two other cAMP agonists 8bromo-cAMP (250  $\mu$ M) and rolipram (10  $\mu$ M), a specific type 4 phosphodiesterase inhibitor, and found that COX-2 mRNA expression was significantly increased at 6 hrs of 8bromo-cAMP treatment (Fig. 1B) and at 1 and 6 hrs of rolipram treatment (Fig. 1C). We then performed a dose–response study and confirmed that forskolin increased in COX-2 mRNA at 6 hrs (100  $\mu$ M,  $P < 0.05$  only; Fig. 1D). This was associated with increased COX-2 protein synthesis at 6 and 24 hrs (Fig. 1F and G) and increased levels of PGE<sub>2</sub>, PGI<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  at 24 and 48 hrs (Fig. 1H–J). In future experiments, we used the dose of 100  $\mu$ M of forskolin and the 6-hr time point to assess both COX-2 mRNA expression and protein synthesis.

### Intracellular mechanisms involved in cAMP-induced COX-2 synthesis

#### Role of PKA, EPAC, AMPK and PDZ-GEF<sub>1,2</sub>

Initially we attempted to identify which cAMP effector (PKA, EPAC or AMPK) was responsible for the cAMP-induced increase in COX-2 mRNA expression. Human myometrial cells were treated with forskolin (100  $\mu$ M) and various cAMP effector activators: PKA

(Sp-6-phe-cAMP, 100 nM), EPAC (8pCPT-2'-O-Me-cAMP, 50  $\mu$ M) and AMPK (AICAR, 100  $\mu$ M). Forskolin increased COX-2 mRNA expression ( $P < 0.05$ ; Fig. 2A) and protein synthesis ( $P < 0.01$ ; Fig. 2B), but none of the cAMP effector activators reproduced these results (Fig. 2A and B).

We then incubated human myometrial cells with forskolin (100  $\mu$ M) alone or in the presence of cAMP effector antagonists: PKA (KT5720, 10  $\mu$ M), EPAC (brefeldin A, 100  $\mu$ M), AMPK (compound C, 100  $\mu$ M). None of the inhibitors blocked the forskolin-induced increase in COX-2 mRNA expression, promoter activity or protein synthesis (Fig. 3A–C).

To confirm the activator/antagonist data, we knocked down the expression of the cAMP effectors, using shRNA to PKA, EPAC and AMPK. In addition, we knocked down PDZ-GEF<sub>1,2</sub>, another potential cAMP effector without known chemical activators or antagonists. Western analysis for PKA, AMPK, EPAC and PDZ-GEF<sub>1,2</sub> showed the efficiency of protein expression inhibition was approximately 80% (Fig. 4A–E). Knockdown of PKA, EPAC, AMPK and PDZ-GEF<sub>1,2</sub> did not inhibit the cAMP-induced increase in COX-2 mRNA, promoter activity or COX-2 protein synthesis (Fig. 4F–H).

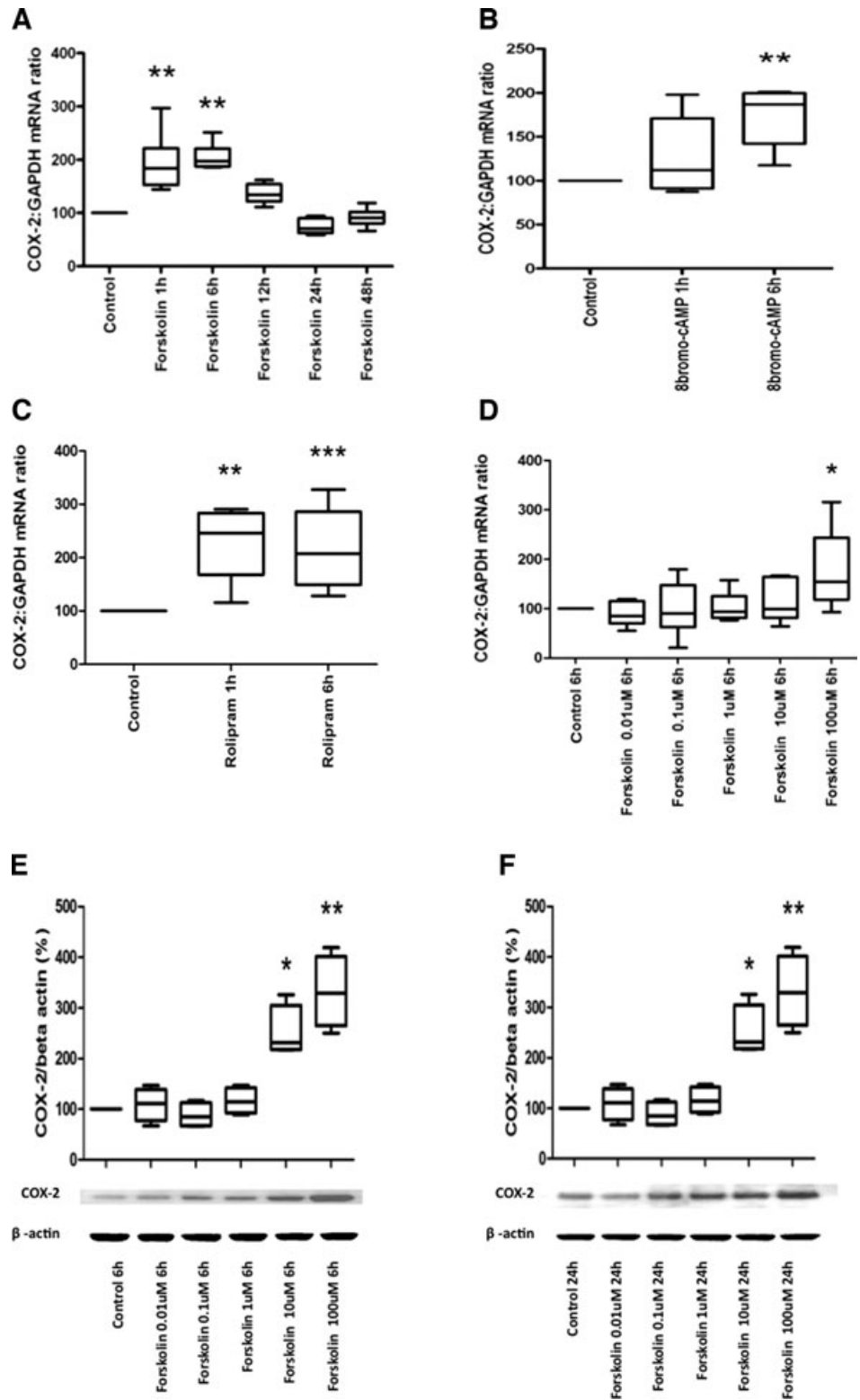
We confirmed that forskolin activated PKA and AMPK by using phospho-CREB and phospho-ACC (Fig. S1A and B). We used phospho-AKT as marker of EPAC activation, but found no increase of phosphorylation (Fig. S1C); to confirm that EPACs were present in human myometrial cells we assessed p38 phosphorylation with the EPAC agonist. In addition, we confirmed that the antagonists of PKA and AMPK were effective by co-incubating with KT5720 (PKA inhibitor, 10  $\mu$ M) and compound C (AMPK inhibitor, 100  $\mu$ M; Fig. S1A and B); we were unable to confirm that the EPAC antagonist, brefeldin A (EPAC inhibitor, 100  $\mu$ M), was effective as we did not see any increase in AKT phosphorylation. We confirmed that the chemical stimulators of the cAMP effectors were active by assessing their respective down-stream substrates, for PKA (Sp-6-phe-cAMP, 100 nM) down-stream substrate phospho-CREB, for AMPK (AICAR, 100  $\mu$ M), down-stream substrate phospho-ACC and for EPAC (8pCPT-2'-O-Me-cAMP, 50  $\mu$ M), down-stream substrate p38 (Fig. S2A–C).

We also investigated the role of PKG in mediating cAMP action because it has been recently recognized that cAMP can act *via* PKG to induce biological effects. Co-incubation of human myometrial cells with forskolin and a PKG inhibitor (Rp-8-Bromo- $\beta$ -phenyl-1,N2-ethenoguanosine 3':5'-cyclic monophosphorothioate sodium salt hydrate, 15  $\mu$ M) had no effect on cAMP-driven COX-2 expression (Fig. S3A).

#### Role of MAPKs

It is well-established that myometrial COX-2 expression is regulated by MAPK-dependent pathways [16]. Consequently, we next assessed whether cAMP-increased COX-2 expression in human myometrial cells involves MAPK activation. We found that forskolin (100  $\mu$ M) significantly increased the

**Fig. 1 (A–E)** Cyclic AMP exposure increases COX-2 mRNA expression, protein synthesis and activity. Human myometrial cells were treated with Forskolin (100 mM) for 0, 1, 6, 12, 24 and 48 hrs, and 8bromo-cAMP (250  $\mu$ M) and rolipram (10 mM) for 0, 1 and 6 hrs ( $n = 6$  in each study). mRNA was extracted, the levels of COX-2 mRNA measured using quantitative rtPCR. COX-2 mRNA expression was increased by forskolin (ANOVA  $P < 0.0001$ , Dunnett's test  $P < 0.01$  at 1 and 6 hrs; **A**), 8bromo-cAMP (ANOVA  $P = 0.008$ , Dunnett's test  $P < 0.01$  at 6 hrs; **B**) and rolipram (Friedman's test = 0.0008, Dunnett's test  $P < 0.01$  at 1 hrs and  $P < 0.001$  at 6 hrs; **C**). Data are shown as the median, the 25th and 75th percentiles and the range. \* $P < 0.05$  and \*\* $P < 0.01$  for control versus treatment. (**D–F**) In separate experiments, human myometrial cells were treated with forskolin 0.01, 0.1, 1.0, 10 and 100 mM for 6 hrs for COX-2 mRNA (measured by qPCR) and 6 and 24 hrs for COX-2 protein levels (measured by Western analysis). At 6 hrs, forskolin increased COX-2 mRNA expression (Friedman's = 0.02, Dunnett's test: 100  $\mu$ M  $P < 0.05$ ; **D**). COX-2 protein levels were increased by forskolin at 6 and 24 hrs (for 6 hrs, ANOVA  $P < 0.0001$ , Dunnett's test: 100 and 10  $\mu$ M  $P < 0.01$ ; **E**, and for 24 hrs, ANOVA  $P < 0.0001$ , Dunnett's test: 100 and 10  $\mu$ M  $P < 0.01$ ; **F**). Data are shown as the median, the 25th and 75th percentiles and the range. \* $P < 0.05$  and \*\* $P < 0.01$  for control versus forskolin. (**G–I**) In separate experiments, human myometrial cells were treated with forskolin 0.1, 1.0, 10 and 100 mM for 6, 24 and 48 hrs the medium was collected and the levels of PGE<sub>2</sub>, PGI<sub>2</sub> and PGF<sub>2a</sub> measured with ELISA as described in the Materials and methods (**G–I**,  $n = 3$ ). The data were analysed using ANOVA, with a Dunnett's *post-hoc* test. Data are shown as the mean; \* $P < 0.05$  and \*\*\* $P < 0.001$ . The statistical significance is shown for the forskolin 100  $\mu$ M concentration.



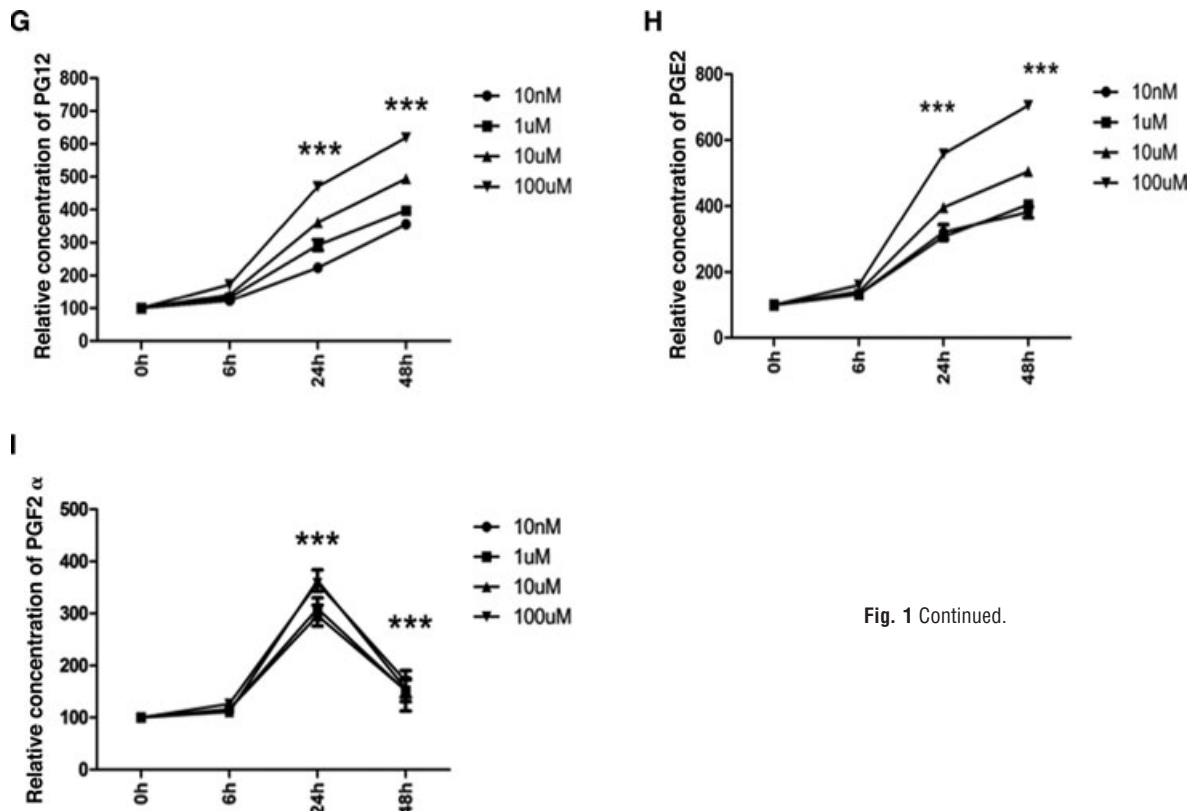
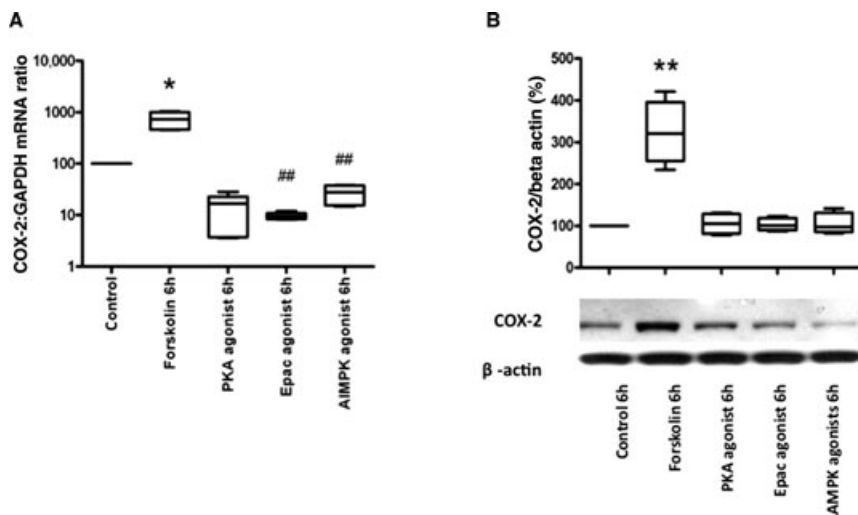


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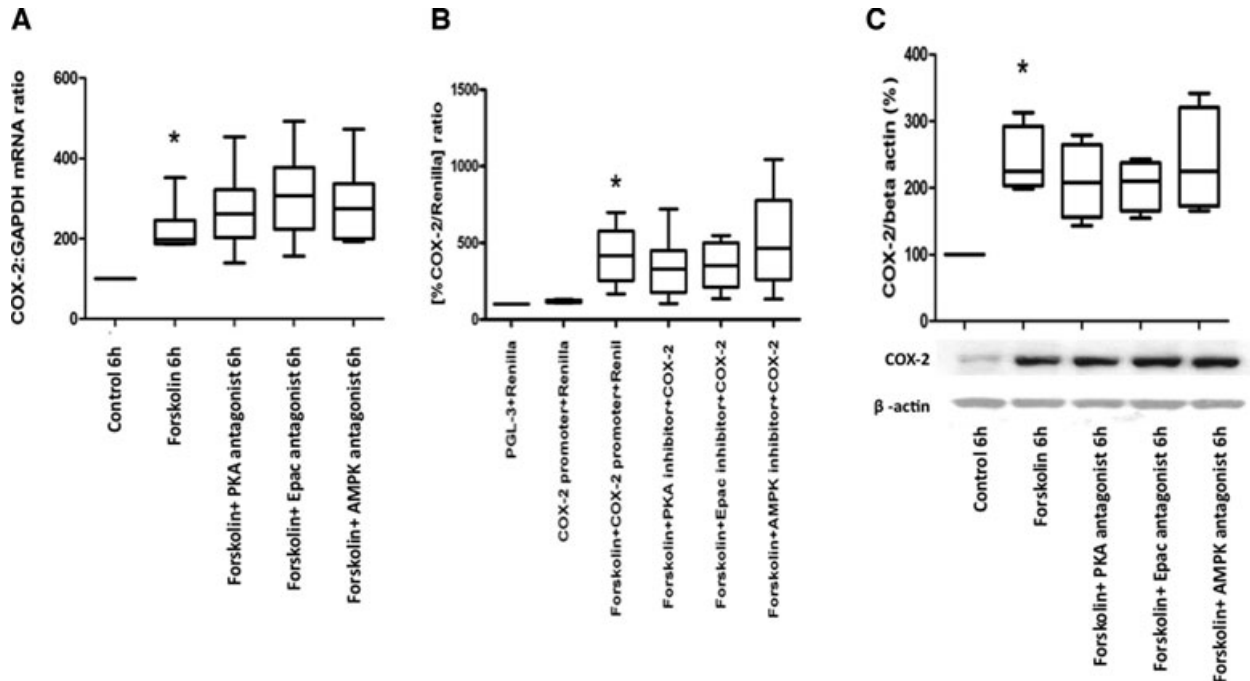


**Fig. 2** Cyclic AMP effector agonists do not reproduce the forskolin-induced increase in COX-2 mRNA expression and protein synthesis. Human uterine smooth muscle cells were treated with forskolin (100  $\mu$ M) and different cAMP mediator agonists, Sp-6-phe-cAMP (PKA agonist) 100 nM, 8pCPT-2'-O-Me-cAMP (Epac agonist) 50  $\mu$ M, AICAR (AMPK agonist) 100  $\mu$ M for 6 hrs; mRNA and protein were extracted, the COX-2 mRNA levels measured using quantitative rtPCR (**A**) and the protein concentration assessed using western analysis (**B**). Control *versus* forskolin mRNA data were analysed using Wilcoxon matched pairs for paired data and Friedman's test, with a Dunn's multiple comparisons *post-hoc* test for analysis of three groups or more. The Western blot densitometry were analysed

with an ANOVA followed by Bonferroni's post-test. Data are shown as the median, the 25th and 75th percentiles and the range. \* $P < 0.05$  and \*\* $P < 0.01$  for control *versus* forskolin and ## $P < 0.01$  for forskolin *versus* cAMP agonists.

phosphorylation of p38 and ERK1/2 for up to 60 min. (Fig. 5A and B) and of JNK for 30 min. (Thr183 and Tyr185; Fig. 5C). Subsequently, we used specific inhibitors of MAPK activity and

found that the forskolin-induced increases in COX-2 mRNA expression, COX-2 promoter activity and COX-2 protein synthesis were reduced (Fig. 5D-F).



**Fig. 3** Cyclic AMP effector antagonists do not reduce forskolin-induced increase in COX-2 mRNA expression, promoter activity or protein synthesis. Human myometrial cells were treated with forskolin (100  $\mu$ M) and different cAMP mediator antagonists, KT5720 10  $\mu$ M (PKA inhibitor), brefeldin A 100  $\mu$ M (Epac inhibitor) and compound C 100  $\mu$ M (AMPK inhibitor) for 6 hrs, mRNA and protein were extracted, the COX-2 mRNA levels measured using quantitative rtPCR (**A**,  $n = 6$ ) and the protein concentration assessed using Western analysis (**C**,  $n = 4$ ). Human myometrial cells were transiently transfected with a COX-2 promoter construct and treated with forskolin in the presence and absence of cAMP-effector antagonists (as detailed earlier), after a 24 hrs the luciferase activity was measured (**B**,  $n = 6$ ). The control *versus* forskolin mRNA data were analysed using Wilcoxon matched pairs for paired data and Friedman's test, with a Dunn's multiple comparisons *post-hoc* test for analysis of three groups or more. The Western blot densitometry and promoter studies were analysed with an ANOVA followed by Bonferroni's post-test. Data are shown as the median, the 25th and 75th percentiles and the range. \* $P < 0.05$  for control *versus* forskolin and  $^{##}P < 0.01$  for forskolin *versus* cAMP antagonists.

## The effect of PGE<sub>2</sub> on COX-2 expression, protein synthesis and promoter activity

To confirm the physiological relevance of these data, we assessed the effect of PGE<sub>2</sub> and found that it increased both COX-2 mRNA (Fig. 6A) and protein (Fig. 6B), acting *via* its EP-2 receptor (Fig. 6C–E). PGE<sub>2</sub> also significantly increased the phosphorylation of p38 (Fig. 7A), ERK (Fig. 7B) and JNK (Fig. 7C). The PGE<sub>2</sub>-induced increases in COX-2 mRNA expression, promoter activity and protein synthesis were reduced in the presence of the MAPK inhibitors (Fig. 7D–F).

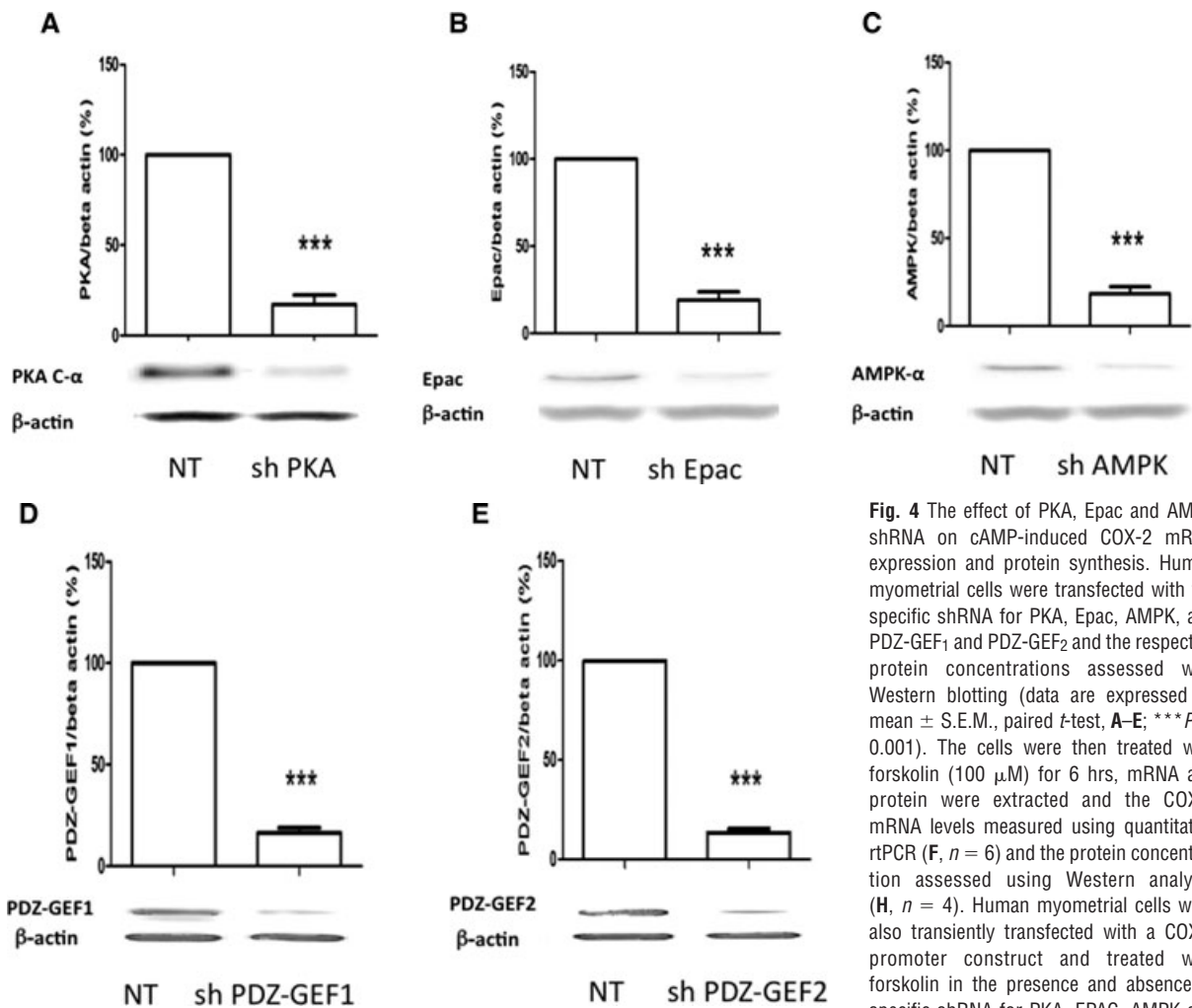
## Discussion

Our data show that cAMP increases the expression, synthesis and activity of COX-2, which is critical to the onset and progression of labour. These data provide more evidence that cAMP has two

opposing effects on human myometrium, on the one hand inducing relaxation and on the other priming the uterus for contraction. These observations are consistent with previous reports that cAMP increases COX-2 mRNA expression in cultures of several human primary cells, including leucocyte, cardiomyocytes, endothelial cells and granulosa cells [20, 21]. Despite the evidence that cAMP increases COX-2 synthesis in other tissues, to prove that this was not an artefact of either the culture system or of forskolin administration, we used other cAMP agonists 8bromo-cAMP and rolipram and performed a dose/time-response study for forskolin.

The recent paper of Fetalvero *et al.* found that cAMP enhanced the synthesis of the contraction associated proteins connexin 43,  $\alpha$ -SMA, h-caldesmon, calponin and SM2-MHC, suggesting that cAMP be important in myometrial activation prior to the onset of labour [19]. In this paper, the effects of cAMP were mediated *via* PKA as shown by the inhibitory effect of PKA knock-down [19]. In other tissues, cAMP has been shown to act *via* PKA (osteoblasts, Ref. 22) and AMPK expression (renal podocytes, Ref. 23) to increase COX-2 expression and to act *via* EPAC to suppress lipopolysaccharide (LPS)-induced interferon expression in





**Fig. 4** The effect of PKA, Epac and AMPK shRNA on cAMP-induced COX-2 mRNA expression and protein synthesis. Human myometrial cells were transfected with the specific shRNA for PKA, Epac, AMPK, and PDZ-GEF<sub>1</sub> and PDZ-GEF<sub>2</sub> and the respective protein concentrations assessed with Western blotting (data are expressed as mean  $\pm$  S.E.M., paired *t*-test, **A–E**; \*\*\**P* < 0.001). The cells were then treated with forskolin (100  $\mu$ M) for 6 hrs, mRNA and protein were extracted and the COX-2 mRNA levels measured using quantitative rtPCR (**F**, *n* = 6) and the protein concentration assessed using Western analysis (**H**, *n* = 4). Human myometrial cells were also transiently transfected with a COX-2 promoter construct and treated with forskolin in the presence and absence of specific shRNA for PKA, EPAC, AMPK and

PDZ-GEF<sub>1&2</sub>, after 24 hrs the luciferase activity was measured (**G**, *n* = 6). The control *versus* forskolin mRNA data were analysed using Wilcoxon matched pairs for paired data and Friedman's test, with a Dunn's multiple comparisons *post-hoc* test for analysis of three groups or more. The Western blot densitometry and promoter studies were analysed with an ANOVA followed by Bonferroni's *post*-test. Data are shown as the median, the 25th and 75th percentiles and the range. \**P* < 0.05 and \*\**P* < 0.01 for control *versus* forskolin and #*P* < 0.05 for forskolin *versus* cAMP effector shRNA.

macrophages [24]. However, using a combination of chemical inhibitors and activators, complimented by knock-down using shRNA, we showed that cAMP in our system was acting independent of known effectors. Given that we have previously shown that the MAPK system is essential for both IL-1 $\beta$  and stretch-induced COX-2 mRNA expression [25], we assessed whether cAMP could be acting *via* MAPK. We found that cAMP induced a robust activation of all MAPK isoforms and that MAPK inhibition blocked the cAMP-induced increase in COX synthesis. This suggested that the cAMP could be working through a guanine nucleotide exchange factor (GEF) to activate MAPK. cAMP-dependent Ras/MAPK activation has been reported in many cell types, but the Ras activator involved was not always identified [26]. In some

reports, EPAC was suggested to have a role [27], but we had already excluded a role for EPAC in our studies. In melanoma cells, PDZ-GEF (also known as RAGEF, nRapGEP or CNrasGEF) was reported to mediate the cAMP activation of Ras/Erk pathway, leading to alterations in melanogenesis [28]. However, when we knocked down PDZ-GEF, we again found that the forskolin induced changes in COX-2 mRNA expression were unaltered. These data suggest that cAMP up-regulates COX-2 expression *via* MAPK, but that the exact pathway involved is as yet unidentified.

Most data suggest that cAMP stimulates the COX-2 promoter *via* the CRE *via* either CREB or AP-1 proteins [29–31], both of which can be activated by MAPK [32]. MAPK activation can also prolong COX-2 mRNA half-life [32]. These effects seem to be

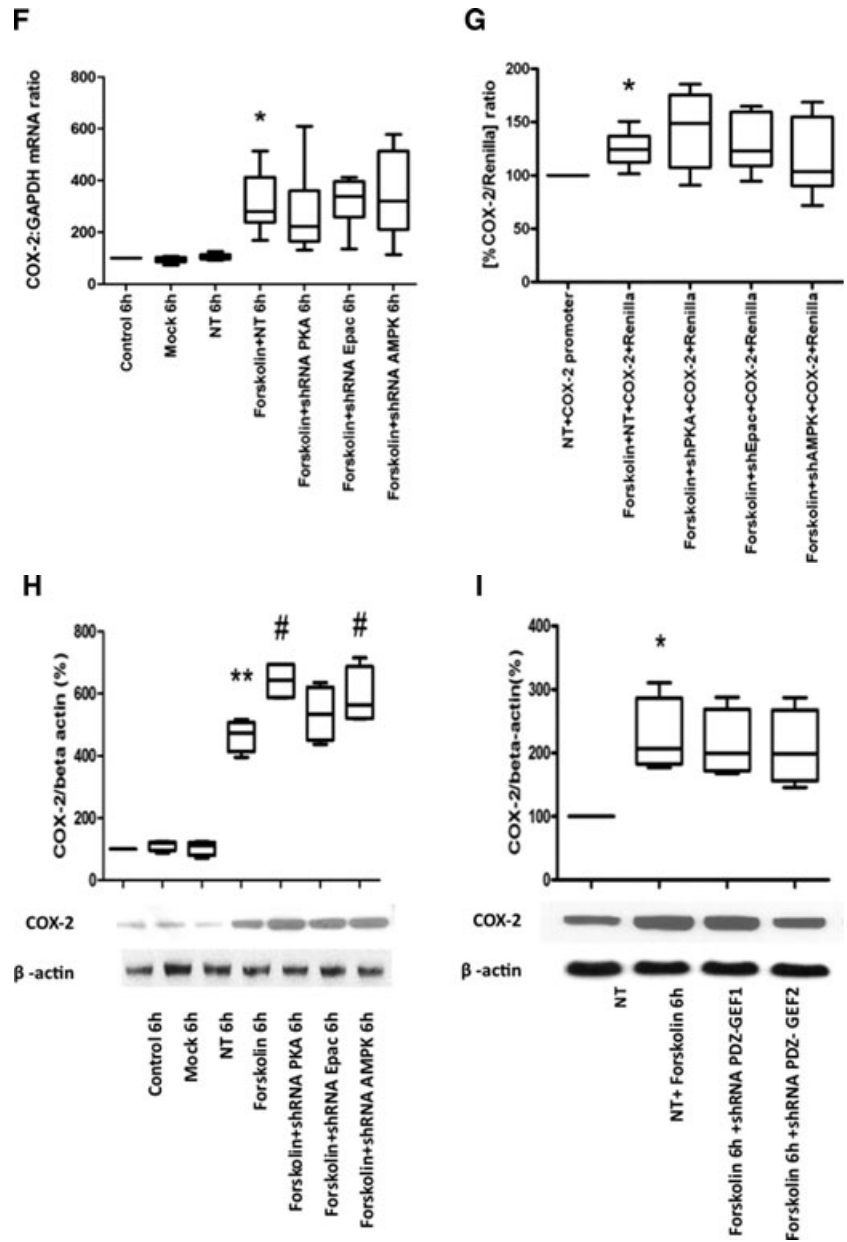
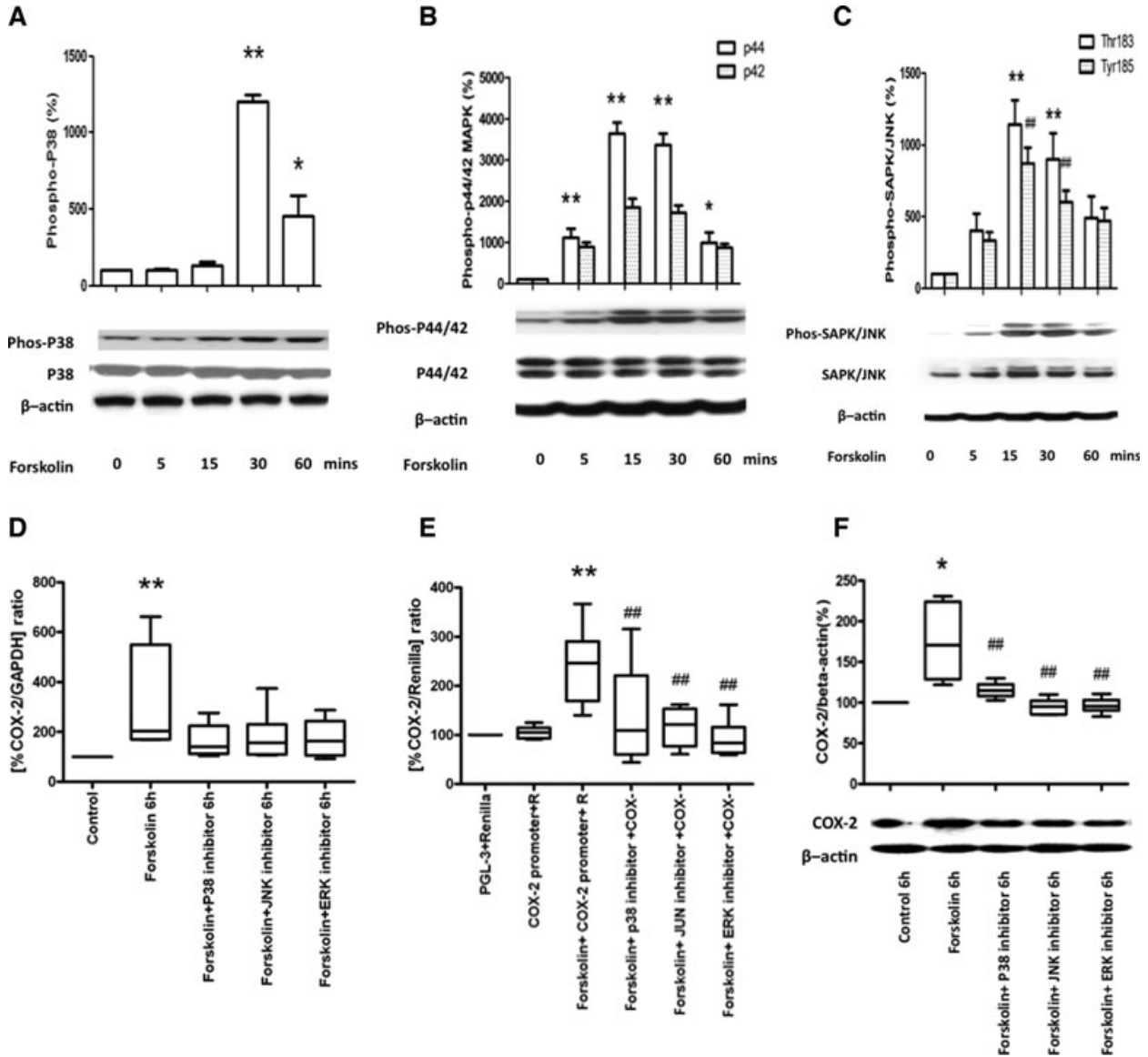


Fig. 4 Continued.

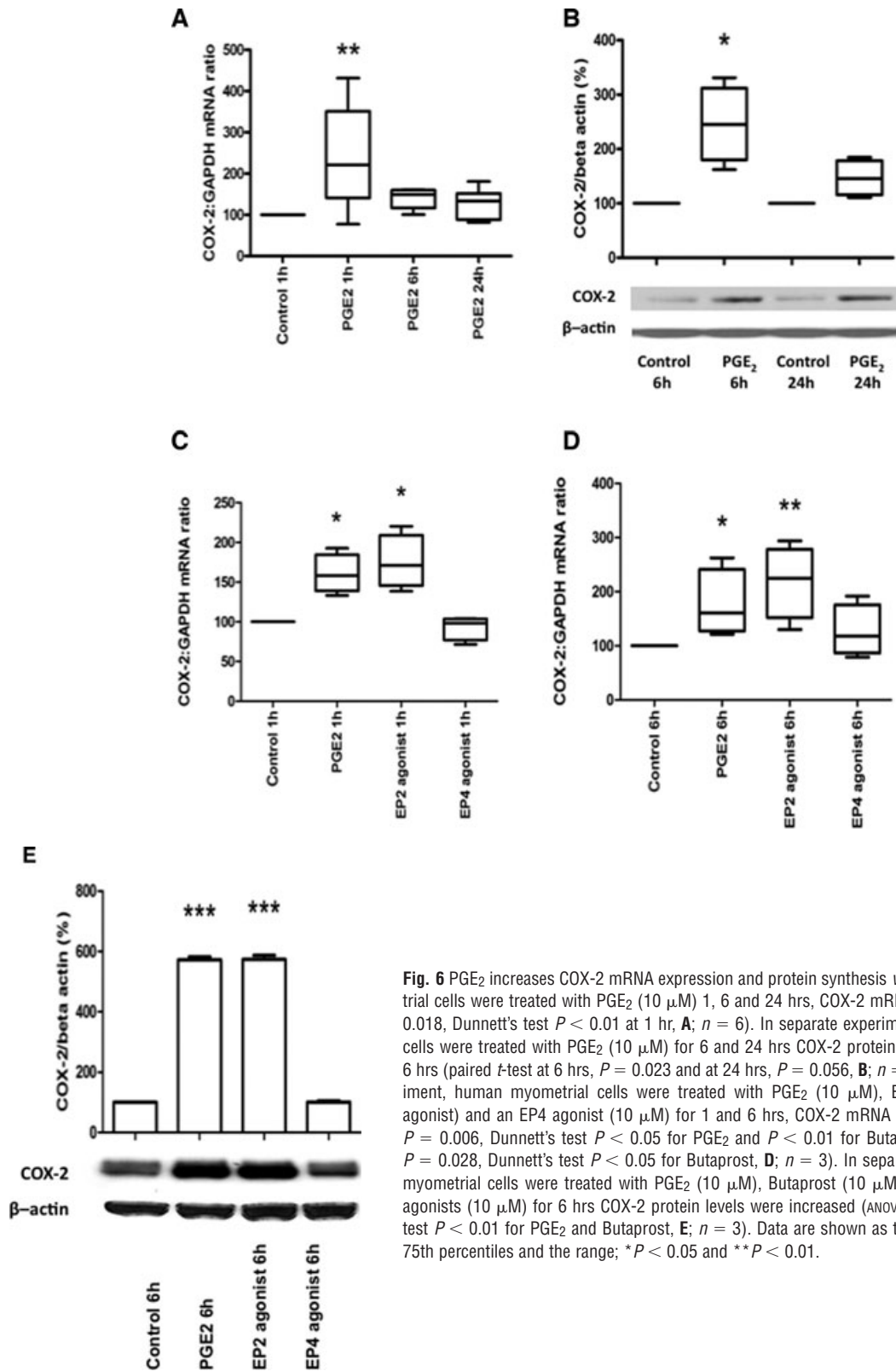
isoform specific, because transforming growth factor- $\alpha$  increased COX-2 mRNA in epidermal keratinocytes *via* ERK-induced increases in transcription and p38-induced increases in mRNA half-life [33]. We observed an increase in COX-2 mRNA associated with increased COX-2 promoter activity with a greater increase in COX-2 protein. In mouse cardiac smooth muscle cells, activation of MAPK-activated protein kinase-2 (MK2), a kinase downstream of p38, increased COX-2 protein levels with out altering COX-2 mRNA levels or protein stability [34], which is consistent with our observations. Interestingly, we found that COX-2 protein remained

elevated after COX-2 mRNA had returned to baseline probably reflecting the stability of COX-2 protein.

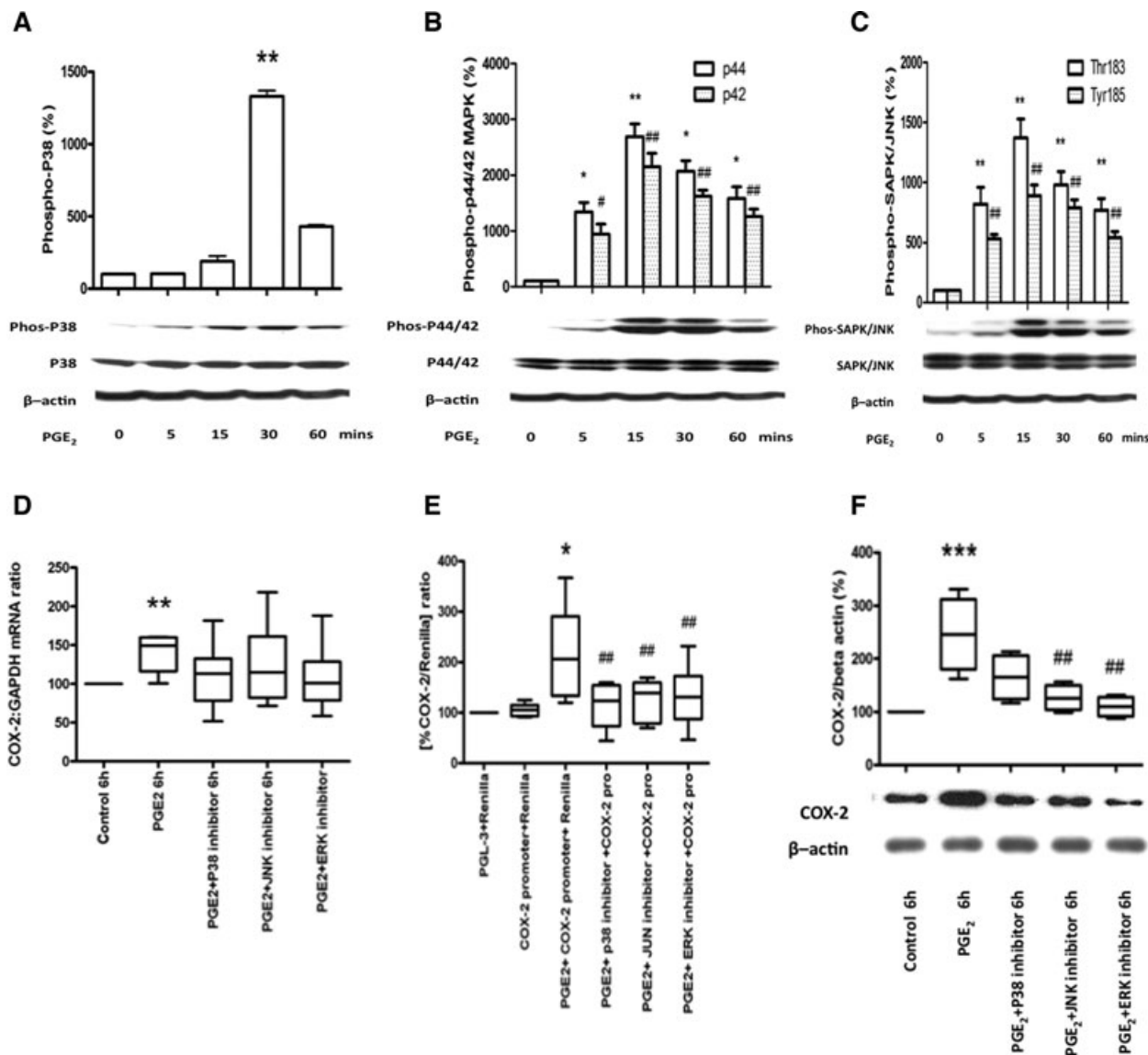
Most studies suggest that cAMP increases COX-2 expression typically on the background of an inflammatory stimulus. A recent review concluded that cAMP should be considered as a positive modulator of COX-2 expression, rather than an inducer in its own right and suggested that the clinical use of  $\beta$ 2-mimetics or PG receptor agonists would not impact on prostanoid synthesis [35]. However, our data show that cAMP and PGE<sub>2</sub> (*via* the cAMP linked EP-2 receptors) have a robust effect on COX-2 expression and



**Fig. 5** MAPK mediates the cAMP-induced increase in COX-2 mRNA expression, protein synthesis and promoter activity. (A–C) Human myometrial cells were treated with forskolin (100  $\mu$ M) for 0, 5, 15, 30 and 60 min. Protein was extracted and Western analysis performed for the phosphoforms of p38, ERK1/2 and JNK, followed by densitometry. Forskolin increased p38 phosphorylation (ANOVA  $P < 0.0001$ , Dunnett's test at 30 and 60 min.  $P < 0.01$  and  $P < 0.05$  respectively, **A**), ERK phosphorylation (for p44, ANOVA  $P < 0.0001$ , Dunnett's test at 5, 15 and 30 min.  $P < 0.01$  and at 60 min.  $P < 0.05$ , p42 ANOVA not significant, **B**) and JNK phosphorylation (for Thr183 ANOVA  $P = 0.0012$ , Dunnett's test at 15 and 30 min.  $P < 0.01$ , for Tyr185 ANOVA  $P = 0.0003$ , Dunnett's test at 15 and 30 min.  $P < 0.01$ , **C**). The data are expressed as mean  $\pm$  S.E.M. \* $P < 0.05$  and \*\* $P < 0.01$  for p38, ERK-p44 and JNK-threonine 183 and ## $P < 0.01$  for JNK-tyrosine 185. For all  $n = 6$ . (D–G) Human myometrial cells were treated with forskolin (100  $\mu$ M) in the presence and absence of specific MAPK inhibitors (p38 inhibitor SB203580 10  $\mu$ M, ERK inhibitor U0126 10  $\mu$ M, JNK inhibitor SP600125 20  $\mu$ M) for 6 hrs. The increases in COX-2 mRNA expression (ANOVA  $P = 0.011$ , Bonferroni's, control versus forskolin  $P < 0.01$ ; **D**,  $n = 6$ ), in COX-2 promoter activity (ANOVA  $P < 0.0001$ , Bonferroni's, control versus forskolin  $P < 0.001$  and forskolin versus forskolin and p38, ERK and JNK inhibitors  $P < 0.001$ ; **E**,  $n = 6$ ), and in COX-2 protein synthesis (ANOVA  $P = 0.0013$ , Bonferroni's, control versus forskolin  $P < 0.05$  and forskolin versus forskolin and p38, ERK and JNK inhibitors  $< 0.01$  at 6 hrs; **F**,  $n = 6$ ) were inhibited. Data are shown as the median, the 25th and 75th percentiles and the range. \* $P < 0.05$  and \*\* $P < 0.01$  between control and forskolin treatment and # $P < 0.05$ , ## $P < 0.01$  between forskolin alone and in the presence of MAPK inhibitors.



**Fig. 6** PGE<sub>2</sub> increases COX-2 mRNA expression and protein synthesis *via* EP-2. Human myometrial cells were treated with PGE<sub>2</sub> (10 μM) 1, 6 and 24 hrs, COX-2 mRNA increased (ANOVA  $P = 0.018$ , Dunnett's test  $P < 0.01$  at 1 hr, **A**;  $n = 6$ ). In separate experiments, human myometrial cells were treated with PGE<sub>2</sub> (10 μM) for 6 and 24 hrs COX-2 protein levels were increased at 6 hrs (paired  $t$ -test at 6 hrs,  $P = 0.023$  and at 24 hrs,  $P = 0.056$ , **B**;  $n = 4$ ). In a separate experiment, human myometrial cells were treated with PGE<sub>2</sub> (10 μM), Butaprost (10 μM, EP-2 agonist) and an EP<sub>4</sub> agonist (10 μM) for 1 and 6 hrs, COX-2 mRNA increased (at 1 hr, ANOVA  $P = 0.006$ , Dunnett's test  $P < 0.05$  for PGE<sub>2</sub> and  $P < 0.01$  for Butaprost, **C**; at 6 hrs, ANOVA  $P = 0.028$ , Dunnett's test  $P < 0.05$  for Butaprost, **D**;  $n = 3$ ). In separate experiments, human myometrial cells were treated with PGE<sub>2</sub> (10 μM), Butaprost (10 μM, EP-2 agonist) and EP<sub>4</sub> agonists (10 μM) for 6 hrs COX-2 protein levels were increased (ANOVA  $P = 0.0005$ , Dunnett's test  $P < 0.01$  for PGE<sub>2</sub> and Butaprost, **E**;  $n = 3$ ). Data are shown as the median, the 25th and 75th percentiles and the range; \* $P < 0.05$  and \*\* $P < 0.01$ .



**Fig. 7** MAPK mediates the PGE<sub>2</sub>-induced increase in COX-2 mRNA expression, protein synthesis and promoter activity. Human myometrial cells were treated with PGE<sub>2</sub> (10 μM), which increased the phosphorylation of p38 at 30 min. (ANOVA  $P < 0.0001$ , Dunnett's test at 30 min.  $P < 0.01$ , **A**), ERK at 5, 15, 30 and 60 min. (p44, ANOVA  $P = 0.0028$ , Dunnett's test at 5 min.  $P < 0.05$ , at 15 and 30 min.  $P < 0.01$  and at 60 min.  $P < 0.05$ ; p42 ANOVA  $P = 0.0003$ , Dunnett's test at 5 min.  $P < 0.05$  and at 15, 30 and 60 min.  $P < 0.01$ , **B**) and JNK at 5, 15, 30 and 60 min. (Thr183 ANOVA  $P = 0.0005$ , Dunnett's test at 5, 15, 30 and 60 min.  $P < 0.01$ ; Tyr185 ANOVA  $P < 0.0001$ , Dunnett's test at 5, 15, 30 and 60 min.  $P < 0.01$ , **C**). The data are expressed as mean  $\pm$  S.E.M. \* $P < 0.05$  and \*\* $P < 0.01$  between control and PGE<sub>2</sub> treatment for p38, ERK-p44 and JNK-threonine 183; # $P < 0.05$  and ## $P < 0.01$  for ERK-p42 and JNK-tyrosine 185. In all cases  $n = 6$ . (**D–F**) In separate experiments, the studies were repeated in the presence and absence of specific MAPK inhibitors (p38 inhibitor SB203580 10 μM, ERK inhibitor U0126 10 μM, JNK inhibitor SP600125 20 μM) for 6 hrs, mRNA and protein were extracted and the COX-2 mRNA levels measured using quantitative rtPCR (**D**,  $n = 6$ ) and the protein concentration assessed using Western analysis (**F**,  $n = 6$ ). Human myometrial cells were also transiently transfected with a COX-2 promoter construct and treated with forskolin in the presence and absence of specific MAPK inhibitors after 24 hrs the luciferase activity was measured (**E**,  $n = 6$ ). The MAPK inhibitors reduced the PGE<sub>2</sub>-induced increase in COX-2 mRNA (**D**), in COX-2 promoter activity (ANOVA  $P = 0.0005$ , Bonferroni's, control versus PGE<sub>2</sub>  $P < 0.001$  and PGE<sub>2</sub> versus PGE<sub>2</sub> with p38  $P < 0.001$ , ERK and JNK inhibitors  $P < 0.01$ ; **E**) and in COX-2 protein (6 hrs: ANOVA  $P = 0.0009$ , Bonferroni's, control versus PGE<sub>2</sub>  $P < 0.001$  and PGE<sub>2</sub> versus PGE<sub>2</sub> with ERK and JNK inhibitors  $P < 0.01$ ; **F**). Data are shown as the median, the 25th and 75th percentiles and the range. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  between control and PGE<sub>2</sub> treatment and ## $P < 0.01$  and ### $P < 0.001$  between PGE<sub>2</sub> alone versus in the presence of MAPK inhibitors.

prostanoid synthesis in human myometrial cells in the absence of an inflammatory stimulus, suggesting that in the myometrium at least, cAMP agonists have a significant effect on prostanoid synthesis. We found that the effect of cAMP was most marked for PGE<sub>2</sub> and least for PGF<sub>2α</sub>. The cAMP-induced increase in both PGE<sub>2</sub> and PGI<sub>2</sub> could establish a positive feedback loop culminating in greater COX-2 expression. Clearly, the effect of cAMP on PG synthesis does not occur in isolation and, as cAMP agonists reduce spontaneous myometrial contractility, at least acutely [36], and rolipram pre-treatment reduces PTL in the mouse LPS model [37], it seems likely the overall effect of an increase in cAMP is to reduce the risk of PTL. However, our data taken with those of Fetalvero *et al.* [19], suggest that cAMP can also activate the myometrium and it is possible that the balance of these opposing effects determines the overall contractile state of human myometrium and may therefore offer an explanation for the limited effect of β-agonists in the treatment of threatened PTL. Thus, although it is accepted that cAMP induces myometrial relaxation acutely, through non-genomic effects, future work must define what its effects are on myometrial contractility in the medium to long-term and the mechanisms involved. This would clarify whether therapies based on increasing intracellular cAMP could be of potential benefit in the management of PTL.

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## Conflict of interests

The authors confirm that there are no conflicts of interest.

## Supporting information

Additional Supporting Information may be found in the online version of this article.

**Fig. S1** Cyclic AMP treatment activated PKA and AMPK in uterine smooth muscle cells.

**Fig. S2** PKA, AMPK and EPAC are activated by chemical stimulators in uterine smooth muscle cells.

**Fig. S3** PKG does not mediate the cAMP-induced increase in COX-2 mRNA expression.

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