

The CRTH2 agonist Pyl A prevents lipopolysaccharide-induced fetal death but induces preterm labour

Lynne Sykes,¹ Bronwen R. Herbert,¹ David A. MacIntyre,¹ Emma Hunte,¹ Sathana Ponnampalam,¹ Mark R. Johnson,¹ Tiong G. Teoh² and Phillip R. Bennett¹

¹Department of Surgery and Cancer, Parturition Research Group, Institute of Reproduction and Developmental Biology, Imperial College London, London, and ²St Mary's Hospital, Imperial College Healthcare NHS Trust, London, UK

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Correspondence: Dr Lynne Sykes, Parturition Research Group, Imperial College Faculty of Medicine, 3rd Floor, Institute of Reproductive and Developmental Biology, Hammersmith Hospital Campus, Du Cane Road, London W120NN, UK.

Email: l.sykes@imperial.ac.uk

Senior author: Phillip R Bennett, email: p.bennett@imperial.ac.uk

Summary

We have previously demonstrated that the anti-inflammatory prostaglandin 15-deoxy- Δ 12,14-prostaglandin J₂ (15dPGJ₂) delays inflammation-induced preterm labour in the mouse and improves pup survival through the inhibition of nuclear factor- κ B (NF- κ B) by a mechanism yet to be elucidated. 15dPGJ₂ is an agonist of the second prostaglandin D₂ receptor, chemoattractant receptor homologous to the T helper 2 cell (CRTH2). In human T helper cells CRTH2 agonists induce the production of the anti-inflammatory interleukins IL-10 and IL-4. We hypothesized that CRTH2 is involved in the protective effect of 15dPGJ₂ in inflammation-induced preterm labour in the murine model. We therefore studied the effects of a specific small molecule CRTH2 agonist on preterm labour and pup survival. An intrauterine injection of lipopolysaccharide (LPS) was administered to CD1 mice at embryonic day 16, \pm CRTH2 agonist/vehicle controls. Mice were killed at 4.5 hr to assess fetal wellbeing and to harvest myometrium and pup brain for analysis of NF- κ B, and T helper type 1/2 interleukins. To examine the effects of the CRTH2 agonist on LPS-induced preterm labour, mice were allowed to labour spontaneously. Direct effects of the CRTH2 agonist on uterine contractility were examined *ex vivo* on contracting myometrial strips. The CRTH2 agonist increased fetal survival from 20 to 100% in LPS-treated mice, and inhibited circular muscle contractility *ex vivo*. However, it augmented LPS-induced labour and significantly increased myometrial NF- κ B, IL-1 β , KC-GRO, interferon- γ and tumour necrosis factor- α . This suggests that the action of 15dPGJ₂ is not via CRTH2 and therefore small molecule CRTH2 agonists are not likely to be beneficial for the prevention of inflammation-induced preterm labour.

Keywords: 15-deoxy- Δ 12,14-prostaglandin J₂; chemoattractant receptor homologous to the T helper 2 cell; lipopolysaccharide; murine preterm labour; nuclear factor- κ B.

Introduction

Preterm labour is one of the most challenging complications of human pregnancy. Its incidence in the western world remains between 6 and 15% depending on the geography and demographics of the population.¹ It is a heterogeneous condition,² with the only firm causal link being that of infection.³ Despite the increased awareness of the association between infection and inflammation and preterm labour,⁴ there have been limited advances in the treatment and prevention of preterm labour.

Currently, there is a drive to develop anti-inflammatory therapies to not only delay preterm labour, but to prevent the long-term neurological damage thought to be a result of the impact of pro-inflammatory factors on fetal inflammatory response syndrome.

The transcription factor nuclear factor- κ B (NF- κ B), which is classically associated with inflammation, is central to regulating the biochemical pathways involved in both term labour and preterm labour.⁵ The oxytocin receptor and cyclo-oxygenase-2 (COX-2) genes contain NF- κ B response elements in their promoter regions.^{6,7}

The oxytocin receptor mediates oxytocin-induced myometrial contractions through activation of phospholipase C and downstream calcium release from intracellular stores.⁸ The COX-2 enzyme is the rate-limiting step for prostaglandin synthesis, which is responsible for uterine contractions and cervical dilatation. NF- κ B is also involved in the transcriptional regulation of matrix metalloproteinases, including matrix metalloproteinase-9, which are required for remodelling of the extracellular matrix,⁹ leading to cervical ripening and fetal membrane rupture. A positive feed-forward loop also exists from activation of NF- κ B by the pro-inflammatory cytokines and subsequently their transcriptional activation, including tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β).^{5,10,11} Hence, premature activation of NF- κ B by pro-inflammatory cytokines, or activation of the Toll-like receptors as a result of infection and inflammation, can lead to the amplification of the pro-inflammatory response and the transcription of the labour-associated genes via NF- κ B activation, resulting in preterm labour.

Inhibition of NF- κ B is an attractive therapeutic target because apart from inhibiting labour-associated genes involved in uterine contractility, cervical ripening and fetal membrane rupture, it would also target pro-inflammatory cytokine production, which may contribute to the neurological damage seen independently of the effect of prematurity. We have previously shown that 15-deoxy- Δ 12,14-prostaglandin J₂ (15dPGJ₂), an anti-inflammatory cyclopentenone prostaglandin, inhibits NF- κ B activity and COX-2 *in vitro* in both human cultured myocytes and amniocytes.¹² In a murine model of inflammation-induced preterm labour, 15dPGJ₂ delays preterm labour from 20 hr post lipopolysaccharide (LPS) injection to 30 hr post LPS plus 15dPGJ₂ injection. More importantly 15dPGJ₂ improved pup survival from 30% with LPS, to 95% with co-injection of LPS and 15dPGJ₂.¹³ The mechanism by which 15dPGJ₂ inhibits NF- κ B is not entirely understood. The 15dPGJ₂ has more than one ligand, including peroxisome proliferator-activated receptor- γ ¹⁴ and the second prostaglandin D₂ (PGD₂) receptor chemotactant receptor homologous to the T helper 2 cell (CRTH2).¹⁵ We have shown that 15dPGJ₂ does not inhibit NF- κ B via the peroxisome proliferator-activated receptor- γ .¹² Whether CRTH2 plays a role in the mechanism of NF- κ B and COX-2 inhibition by 15dPGJ₂ is currently unknown.

CRTH2 is a G protein-coupled receptor linked to the G_{z β /o} subunit.¹⁶ It is the classical receptor of the T helper type 2 (Th2) cell,¹⁷ and has also been identified on eosinophils¹⁸ and basophils.¹⁹ CRTH2 mRNA has been detected in non-pregnant human uterine tissue,²⁰ placenta and choriondecidua.²¹ Prostaglandin D₂ stimulates the production of the Th2 cytokines IL-4, IL-5, IL-13 and IL-10 in cultured Th2 cells *in vitro*.²² Interleukin-4 is a classic Th2 cytokine that is able to inhibit the Th1 response

directly, with IL-10 inhibiting the production of inflammatory mediators indirectly.²³ Interleukin-10 has also been shown in the mouse to protect the fetus by reducing fetal loss as a result of pro-inflammatory cytokines.²⁴ The function of CRTH2 in non-immune cells remains unclear.

We sought to determine if a small molecule CRTH2 agonist was able to mimic the effects of 15dPGJ₂ by exerting anti-inflammatory effects and subsequently delaying preterm labour and providing neuroprotection for the fetus and increased pup survival. The effect of CRTH2 agonists on murine uterine contractility was examined *ex vivo* using a myograph.

Materials and methods

Reagents

The small molecule agonist CRTH2, referred to from now on as Pyl A, was synthesized commercially by Oxygen Healthcare, (Cambridge, UK) and is chemically identical to the L-888 607 compound from the Merck Frosst Centre for Therapeutic Research (Quebec, QC, Canada).²⁵ The compound has an indole core with an acetic acid side chain and a phenyl sulphide group, which is para-substituted by a chlorine atom. Based on the pharmacokinetics of 5 mg/kg described by Gervais *et al.*,²⁵ 250 μ g of Pyl A was used for intrauterine injection. The CRTH2 antagonist GSKCRTH2X was obtained from Glaxo Smith Kline, (London, UK) and 15dPGJ₂ from Cayman Chemicals (Ann Arbor, MI). *Escherichia coli* LPS serotype 0111: B4 (Sigma, St Louis, MO) was used in the murine model of inflammation-induced preterm labour.

Ethics statement

Human blood from non-pregnant women of childbearing age was collected in accordance with the South East London Ethics Committee approval Ref: 10/H0805/54, and in accordance with Imperial College NHS Healthcare Trust Research and Development department where recruitment took place. All blood was collected with written informed consent. Animal studies were performed under UK Home Office Licence 70/6906 and in accordance with the UK Animals (Scientific Procedures) Act of 1986, and the Imperial College Ethics Review Board.

Flow cytometry of granulocytes for detection of CR3 (CD11b) expression

A protocol based on previous studies on CR3 (CD11b) expression was followed.¹⁵ Four millilitres of human blood was collected in sodium citrate vacutainers and the granulocyte fraction was isolated by incubating 1 : 1 blood : 4.5% Dextran (Fluka Analytical, Sigma,

Gillingham, UK) in PBS for 45 min at 4°. The leucocyte fraction was centrifuged at 500 g for 10 min, and the pellet was resuspended in PBS containing CaCl₂ (0.9 mM) and MgCl₂ (0.5 mM) and counted. Cells were then pre-incubated at 37°, followed by treatment with the CRTH2 agonists Pyl A or 15dPGJ₂ for 15 min. The reaction was terminated by the addition of 1 ml ice-cold FACSFlow. In experiments with the CRTH2 antagonist, pre-incubation with GSKCRTH2X was performed for 10 min at 37°. The cells were then centrifuged at 400 g for 5 min at 4° and resuspended in PBS with 2% fetal calf serum for labelling with phycoerythrin-conjugated anti-CD11b and allophycocyanin-conjugated anti-CD49d for 10 min at 4° in the dark. The red cells were then lysed by the addition of Optilyse-C for 10 min in the dark at room temperature. Cells were then washed and resuspended in PBS and 1% fetal bovine serum for analysis. Eosinophils were identified as CD49d positive and by high side and forward scatter. Flow cytometry settings were as follows: Forward scatter E0 Voltage, 1.00 Amp gain Lin, and Side scatter of 329 Voltage, 1.00 Amp gain Lin.

Murine model of infection-induced preterm labour

CD1 outbred virgin female and stud male mice (Charles River, Margate, UK) were purchased at 6–8 weeks of age. All mice were housed in open cages at 21 ± 1°, on a 12 : 12 light : dark cycle regimen, with *ad libitum* access to standard chow and water. Timed mating was performed, with the presence of a copulatory plug being classed as E0 (day 0) of gestation. A mini-laparotomy was performed on embryonic day 16 (E16) of gestation correlating with human gestation of between 33 and 34 weeks. Morphine analgesia (2.5 mg/kg) was administered subcutaneously 20 min before surgery. Both uterine horns were exteriorized and the number of live fetuses per horn was determined. Twenty micrograms (25 µl total volume) *Escherichia coli* LPS serotype 0111:B4 (Sigma) or sterile PBS was injected into the upper right uterine horn between the first and second sacs taking care not to enter the amniotic cavity. Two-hundred and fifty micrograms of Pyl A or vehicle control was then injected between the second and third sacs. Treatment groups consisted of (i) vehicle, (ii) LPS, (iii) LPS and Pyl A and (iv) Pyl A alone.

Animals were allowed to recover before fetal wellbeing assessment and tissue collection (myometrium and pup brain) at 4.5 hr post injection. A qualitative assessment of fetal viability was made in accordance with Pinto-Machado.²⁶ Fetuses were deemed viable if they were pink and moved spontaneously or in response to stimulus. In subsequent experiments dams were allowed to deliver spontaneously. Continuous monitoring was achieved via a remote infrared CCTV system. A dose—response for the LPS was first performed to obtain the lowest dose at which preterm delivery was consistently obtained. For

tissue harvesting, mice were anaesthetized and killed by cervical dislocation. A laparotomy was performed immediately and pups were killed by decapitation in accordance with the project licence. Before processing tissue, uteri were incised in the longitudinal direction and pups were expelled. Right and left horns of the uterus were snap frozen separately with placentas and vasculature removed. Myometrium from the frozen left uterine horns were used for analysis. Pup brains were also extracted and snap frozen. Tissue was stored at –80° until processing.

Protein extraction

Tissue was ground with a pestle and mortar in liquid nitrogen and homogenized in whole cell lysis buffer (150 mM NaCl, 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, with phosphatase Inhibitor (Sigma) and protease inhibitor (Roche, Burgess Hill, UK). The homogenate was incubated on ice for 5 min and centrifuged for 20 min at 16 200 g at 4°. The supernatant was stored at –80° until use. Protein quantification was performed using the Bio-Rad assay, measuring absorbance at 655 nm (Bio-Rad, Hemel Hemstead, UK).

SDS–PAGE and Western blotting

Approximately 15 µg of extracted protein per sample was resolved by SDS–PAGE and subsequently transferred onto PVDF membranes (GE Healthcare, Little Chalfont, UK) at 100 constant V at 4°. Following transfer, the membrane was then blocked in 5% (weight/volume) milk in Tris-buffered saline with tween (TBST×1) for 1 hr. The membrane was then probed with phospho-p65 (Ser 536) (Cell Signalling, Danvers, MA) primary antibody (1 : 1000 in TBS) overnight at 4° or COX-2 (Santa Cruz, Dallas, TX) primary antibody (1 : 2000 in 1% milk in TBS) for 2.5 hr at room temperature, followed by secondary antibody (1 : 2000 in 1% milk/TBS) for 1 hr at room temperature. Chemiluminescence detection was then carried out with ECL Plus (GE Healthcare). The membranes were developed using a high-performance chemiluminescence film (GE Healthcare). Blots were scanned and densitometry was performed with IMAGEJ (v1.44p).

Detection of CRTH2 and interleukin mRNA

Total RNA was isolated from tissue with Trizol[®] according to the manufacturer's instructions. Tissue was washed in PBS and homogenized using the power homogenizer in 1 ml Trizol[®] per 100 mg of tissue. 1 µg RNA was incubated with 1 µl DNase and 1 µl DNase buffer made up to 10 µl volume with diethylpyrocarbonate-treated water for 15 min at room temperature for removal of contaminating DNA. Eight microlitres of the DNase-treated mix was incubated with 1 µl 10 mM dNTP and

1 μ l oligo-dT(12–18) (0.5 μ g/ml) for 5 min at 65°. To this mix, 2 μ l 10X RT buffer, 4 μ l 25 mM MgCl₂, 2 μ l 0.1 mM dithiothreitol, 1 μ l RNase Out and 1 μ l Superscript III was added. (In the reverse transcriptase controls no Superscript III was added.) The mix was incubated at 42° for 10 min and the reaction was terminated at 70° for 15 min. Then 0.5 μ l RNase H was added and the mix was incubated at 37° for 20 min. Samples were stored at –20° until further use. PCR was used to amplify the cDNA. Paired oligonucleotide primers for amplification of the genes of interest were designed to produce amplicons where the intron/exon boundary was crossed wherever possible. Non-template reverse transcriptase controls were used. Table 1 provides the primers for CRTH2, L-19, COX-2 and the cytokines IL-4, IL-10, interferon- γ (IFN- γ) and TNF- α .

Multi-spot enzyme-linked immunosorbent assay

The mesoscale discovery multi-spot ultrasensitive mouse Th1/Th2 9-plex assay was used as per the manufacturer's protocol for the detection of the following cytokines: IL-12, IFN- γ , TNF- α , IL-1 β , KC/GRO, IL-4, IL-5, IL-10 and IL-2. Cytokines were quantified against an eight-point calibration curve from 0 to 2500 pg/ml, constructed from serially diluted standards provided by the kit. The 96-well multi-spot plate was blocked in 1% BSA in PBS for 1 hr before the addition of 40 μ g of murine myometrium or 100 μ g of pup brain protein lysate and incubated for 2 hr at room temperature. The multi-spot ELISA plate was read using a Sanger 2400 imager. The quantities of cytokines were determined against the standard curve and transferred into an EXCEL spreadsheet for further analysis.

Myometrial contractility

Mice were killed by cervical dislocation at E15–16 of gestation; the uterus was harvested, kept in PBS on ice and was used within 5 hr of harvesting. The uterus was dissected either in the longitudinal or horizontal direction to expel the fetuses and the placentas. Vasculature and

decidua were removed macroscopically, and 5 \times 10 mm strips were mounted on the DMT myograph (DMT, Aarhus, Denmark) in the orientation dependent on the muscle type being examined; longitudinal direction for longitudinal muscle and horizontally for the circular muscle orientation. Strips were stretched to 3 g of tension in the organ baths containing 4 ml Krebs solution (glucose 2.0 g/l, magnesium sulphate 0.141 g/l, potassium phosphate 0.16 g/l, potassium chloride 0.35 g/l, sodium chloride 6.9 g/l, calcium chloride dehydrate 0.373 g/l, sodium bicarbonate 2.1 g/l, pH 7.4) and was gassed with 95% O₂ and 5% CO₂. Tissue was allowed to equilibrate for 30 min. Cumulative dose responses were performed after 30 min of spontaneous contractions were recorded to serve as baseline contractility. At the end of the experiment 10⁻⁷ M oxytocin was added to demonstrate strip viability. Concentrations from 0.1 to 100 μ M were added every 20 min at the time of organ bath wash out. Contractility was analysed using the POWERLAB software V 5.5.6 (ADI instruments, Oxford, UK) using the peak parameters extension. Data were transferred from the datapad of the POWERLAB software onto an EXCEL spreadsheet for analysis. Response to treatment was measured by normalizing to baseline spontaneous contractility and divided by the relevant time-point for the vehicle control.

Statistical analysis

Experimental groups consisted of at least three replicates unless otherwise stated. Statistical analysis was performed with GRAPH-PAD PRISM v5 (GraphPad Software, San Diego, CA). One-way analysis of variance or analysis of variance of repeated measures was conducted, with either Dunnett's or Bonferroni's multiple comparisons tests. Samples with $P < 0.05$ were considered to be statistically significant.

Results

CRTH2 mRNA

CRTH2 mRNA was detected in murine myometrium by RT-PCR, using L-19 as a housekeeping gene. No

Table 1. Primer sequences used for amplification of CRTH2, cyclo-oxygenase 2 (COX-2), L-19 and the cytokines: interleukin-4 (IL-4), IL-10, interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α)

	Forward 5'–3'	Reverse 5'–3'	bp
CRTH2	CGTAGCCGTGAGCCTGCGACTG	GGCGATTGCGGAGCCCACCACT	344
COX-2	AGCCAGGCAGCAAATCCTTGCTGTT	TCAAATCCTGTGCTCATAACATTCC	71
IFN- γ	AGCGGCTGACTGAACTCAGATTGTAG	CGGGTGTAGTCACAGTTTTCAGCTGTATAGG	252
TNF- α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC	175
IL-4	CGAAGAACACCACAGAGAGTGAGCT	GACTCATTCATGGTGCAGCTTATCG	237
IL-10	ACCTGGTAGAAGTGATGCCCCAGGCA	CTATGCAGTTGATGAAGATGTCAAAA	181
L-19	GAAAAAGAAGGTCTGGTTGGA	TGATCTGCTGACGGGAGTTG	72

significant difference in CRTH2 expression was seen between the treatment groups (Fig. 1). Amplification of CRTH2 was seen by cycle 33 and L-19 by cycle 19.

Pyl A up-regulates CR3 (CD11b) via CRTH2

The CRTH2 agonists PGD₂ and 15dPGJ₂ increase the expression of CR3 (CD11b) on eosinophils and basophils via CRTH2.^{15,27} Before experiments with the CRTH2 agonist Pyl A, activity at the CRTH2 receptor was confirmed by demonstrating up-regulation of CR3 (CD11b) in human eosinophils. We used flow cytometry to detect CR3 (CD11b) expression on eosinophils, identified by high intensity CD49d expression and forward and side scatter characteristics (Fig. 2). Up-regulation of CR3 (CD11b) expression with Pyl A treatment was demonstrated by an increase in mean fluorescence intensity of CD11b-PE ($P < 0.01$). This effect was attenuated with previous incubation of cells with the CRTH2 antagonist GSKCRTH2X (Fig. 2a,b). The effect of Pyl A was identical to the effect of 15dPGJ₂ in causing increased expression of CR3 (Fig. 2c).

Pyl A induces preterm labour in the mouse

We sought to determine if the CRTH2 agonist Pyl A had the same tocolytic and feto-protective effect as 15dPGJ₂ in delaying preterm labour in LPS-treated mice. A dose-response effect was demonstrated with LPS (serotype 0111:B4) since varying potencies can be seen between serotypes and within batches.²⁸ Administration of 20 µg LPS led to reliable preterm delivery with the least variation between mice (Fig. 3a). No surviving pups at the time of delivery were seen with concentrations above 10 µg (Fig. 3b). Subsequent experiments were performed with

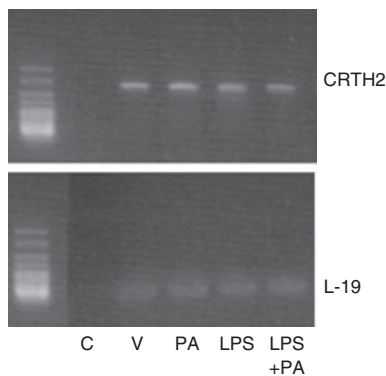


Figure 1. Murine myometrial CRTH2 mRNA. The mRNA was isolated from murine uterus and converted to cDNA ($n = 3$ per treatment group). RT-PCR was used to amplify CRTH2 showing a product size of 344 bp. No difference in CRTH2 expression was seen between treatment groups. CRTH2 mRNA expression was comparable between mice. C = Non-template control, V = vehicle, PA = Pyl A, LPS = lipopolysaccharide.

20 µg LPS. Twenty micrograms of LPS with vehicle or 250 µg Pyl A was given by intrauterine injection under general anaesthetic and mice were allowed to labour spontaneously. Vehicle control mice delivered 64.5 hr post injection and LPS-treated mice delivered 7.7 hr post injection ($P < 0.001$) (Fig. 4a). Co-injection of LPS and Pyl A augmented delivery to 5.8 hr (mean) post injection (Fig. 4a). This effect was more pronounced with a higher dose of Pyl A (500 µg) and lower dose of LPS (10 µg), shortening delivery time from 14.7 to 8.7 hr post injection ($P < 0.01$) (Fig. 4b). Although at 250 µg Pyl A alone did not induce labour, at 500 µg labour was induced at 44.8 hr post injection from 64.6 hr in the vehicle control group. None of the vehicle control-treated mice delivered preterm.

Pyl A prevents LPS-induced intrauterine death

We then determined if the CRTH2 agonist Pyl A maintained the same feto-protective effect as 15dPGJ₂ by examining fetal wellbeing at 4.5 hr post intrauterine injection of LPS with vehicle or Pyl A. Mice were anaesthetized and underwent a caesarean section. Fetuses were assessed for viability by assessment of colour and movement with or without mechanical stimulus. A significant improvement in fetal viability was observed when LPS-treated mice were co-injected with Pyl A compared with LPS and vehicle control. There was a clear difference in the appearance between both groups, in that the LPS-treated mice were clearly dead with no respiratory effort, whereas the LPS/Pyl A-treated mice were pink, moved spontaneously or with stimulus, and had respiratory effort. Fetal survival was increased from 20% in LPS-treated mice to 100% in LPS/Pyl A-treated mice, ($P < 0.0001$) (Fig. 5a). However, following spontaneous labour no pups were viable in the LPS-treated and LPS/Pyl A-treated groups (Fig. 5b).

The effect of Pyl A on the inflammatory cascade in the myometrium and fetal brain

To explore the mechanisms behind Pyl A-augmented LPS-induced preterm labour, key mediators of inflammation in the myometrium were investigated. Myometrium and pup brain were harvested at 4.5 hr post intrauterine injection and Western blotting was used to detect whole cell phospho-p65 and COX-2. Administration of LPS did not lead to an increase in NF-κB in the myometrium; however, an increase was seen with co-administration of LPS and Pyl A ($P < 0.05$) (Fig. 6a). A reduction was seen in NF-κB in pup brain with LPS compared with vehicle control, with no increase with co-administration with Pyl A (Fig. 6b). No significant difference in COX-2 protein expression was seen between treatment groups in the myometrium or pup

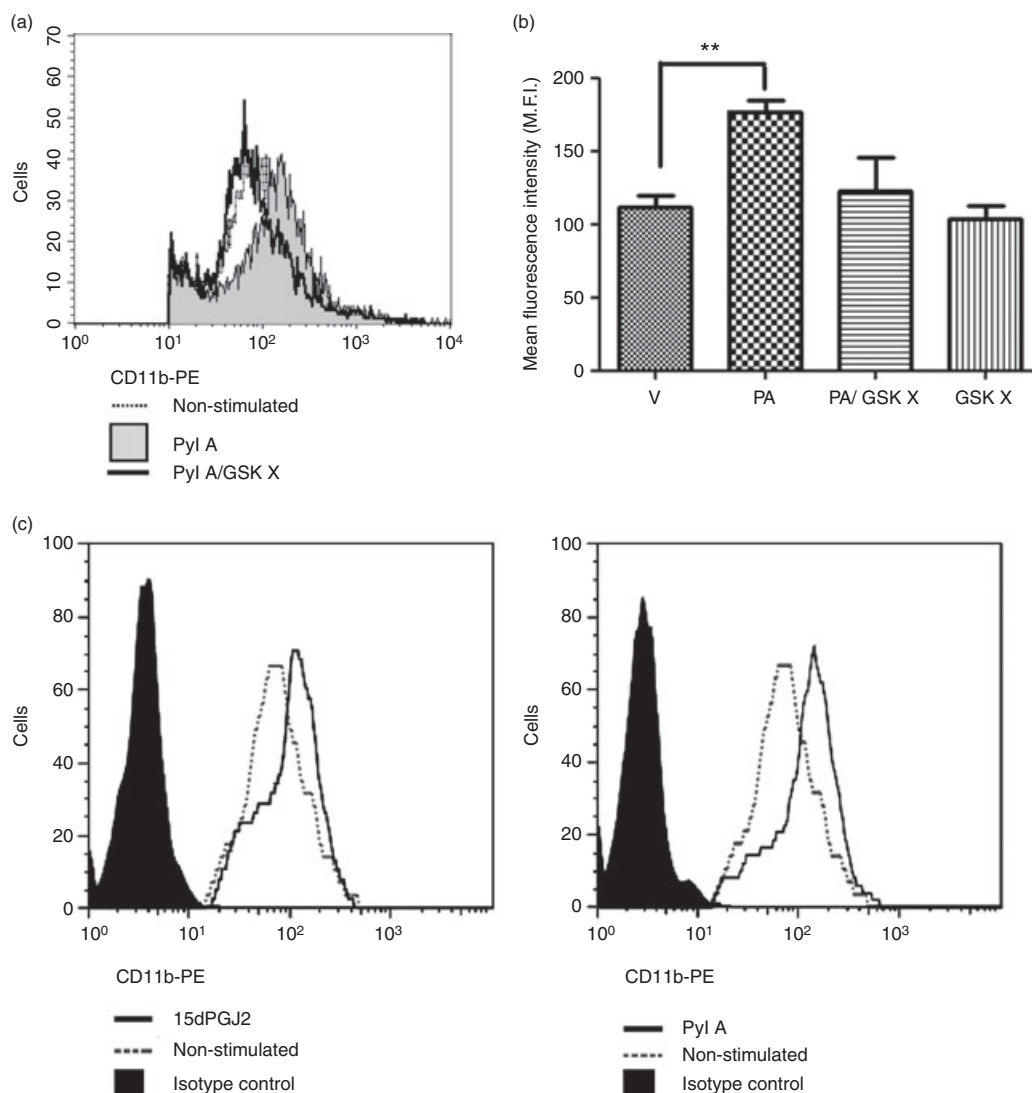


Figure 2. The effect of Pyl A and GSKCRTH2X on CR3 (CD11b) expression on eosinophils. Pyl A (32 μ m) was used to increase CR3 (CD11b) expression on eosinophils. Pre-treatment with the CRTH2 antagonist GSKCRTH2X (100 μ m) was used to confirm this effect was via CRTH2. Eosinophils were identified by labelling with anti-CD49d and on the basis of forward and side scatter. A representative histogram reveals a clear shift to the right with Pyl A treatment indicative of an increase in CR3 (CD11b) expression. This effect was attenuated with CRTH2 antagonist pre-treatment (a). A summary of CR3 (CD11b) expressed in eosinophils with each treatment is shown in the graph ($n = 3$) (b). The effect of Pyl A on CR3 expression is identical to that of 15dPGJ₂. A representative histogram is shown for the effect of both 15dPGJ₂ and Pyl A on CR3 expression (c). V = vehicle, PA = Pyl A, GSK X = GSKCRTH2X. For statistical analysis, analysis of variance of repeated measures with Dunnett's post hoc test was used; ** $P < 0.01$.

brain at this time-point (Fig. 6c,d). However, the messenger RNA of COX-2 was increased in the myometrium of dams treated with Pyl A and LPS compared with other treatment groups (Fig. 6e).

The effect of Pyl A on inflammatory cytokines

We next sought to determine whether activation of NF- κ B resulted in downstream activation of pro-inflammatory cytokines. As the CRTH2 agonist PGD₂ induces the production of the Th2 cytokines IL-10 and IL-4 in human T cells,²² we anticipated that Pyl A would lead to

an increase in these anti-inflammatory cytokines and an inhibition of the pro-inflammatory cytokines. However, consistent with NF- κ B activation, Pyl A and LPS led to an increase in the pro-inflammatory cytokines (Fig. 7). Messenger RNA of the Th1 cytokines IFN- γ and TNF- α was significantly increased at 4.5 hr post injection ($P < 0.05$ and $P < 0.01$, respectively); however, the increase in protein expression did not reach statistical significance. Protein expression levels of other pro-inflammatory cytokines were significantly elevated including IL-1 β , KC/GRO (the murine chemokine equivalent of human IL-8²⁹), and IL-12 ($P < 0.05$).

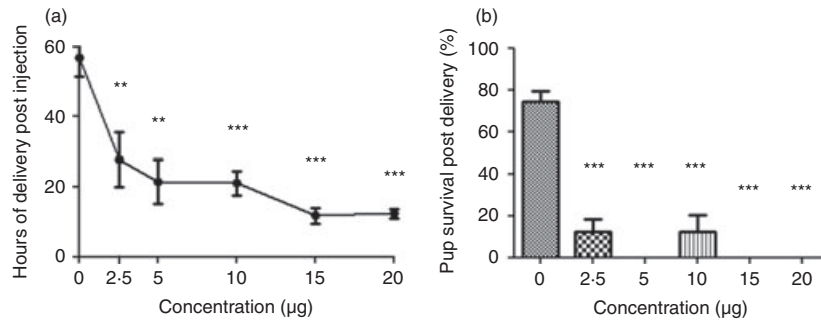


Figure 3. Dose–response of lipopolysaccharide (LPS) induced preterm labour. CD1 mice received an intrauterine injection of vehicle or LPS at E16 and were allowed to deliver, ($n = 3–10$ per treatment group). No vehicle control mice delivered preterm and LPS induced preterm labour in a dose–response effect, (a). No surviving pups were seen at an LPS dose of $>10 \mu\text{g}$ (b). For statistical analysis, one-way analysis of variance with Dunnett’s post hoc test comparing all groups to the vehicle control was used; $**P < 0.01$, $***P < 0.001$.

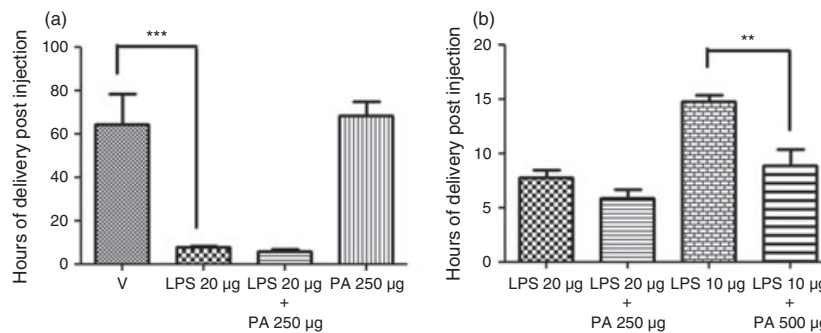


Figure 4. The effect of Pyl A on lipopolysaccharide (LPS) -induced preterm labour. CD1 mice received an intrauterine injection of vehicle, LPS or Pyl A at E16 and were allowed to deliver, ($n = 4$ per treatment group). No vehicle control mice delivered preterm, and at $250 \mu\text{g}$ Pyl A alone did not induce preterm labour (a). LPS induced preterm labour in a dose–response manner, and this effect was augmented with the addition of Pyl A (b). V = vehicle, PA = Pyl A. For statistical analysis, one-way analysis of variance with Bonferroni’s multiple comparison’s test was used; $**P < 0.01$, $***P < 0.001$.

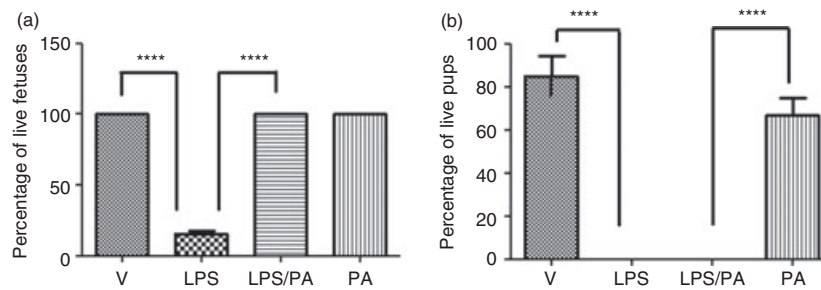


Figure 5. Effect of Pyl A on intrauterine fetal viability at 4.5 hr post intrauterine injection. Dams were killed at 4.5 hr post intrauterine injection of $20 \mu\text{g}$ of lipopolysaccharide (LPS), $250 \mu\text{g}$ of Pyl A and pup viability was assessed ($n = 3$ dams). An average of 11–14 pups per dam was seen in each treatment condition. Pyl A significantly increased viability at 4.5 hr post injection from 20% survival to 100% (a). In a subsequent experiment mice were allowed to deliver spontaneously. No pups in the LPS-treated or LPS/Pyl A-treated groups survived premature delivery (b). V = vehicle, PA = Pyl A. For statistical analysis, one-way analysis of variance with Bonferroni’s multiple comparison test was used; $****P < 0.0001$.

The effect of Pyl A on the anti-inflammatory cytokines

The mechanism of increased *in utero* fetal survival seen with Pyl A was explored by analysing the mRNA and

protein expression of Th2 anti-inflammatory cytokines in the myometrium and pup brains. There was no difference in IL-4 mRNA between treatment groups, and protein concentrations were below the detection level of the assay. There was a slight increase in the production of IL-5, and

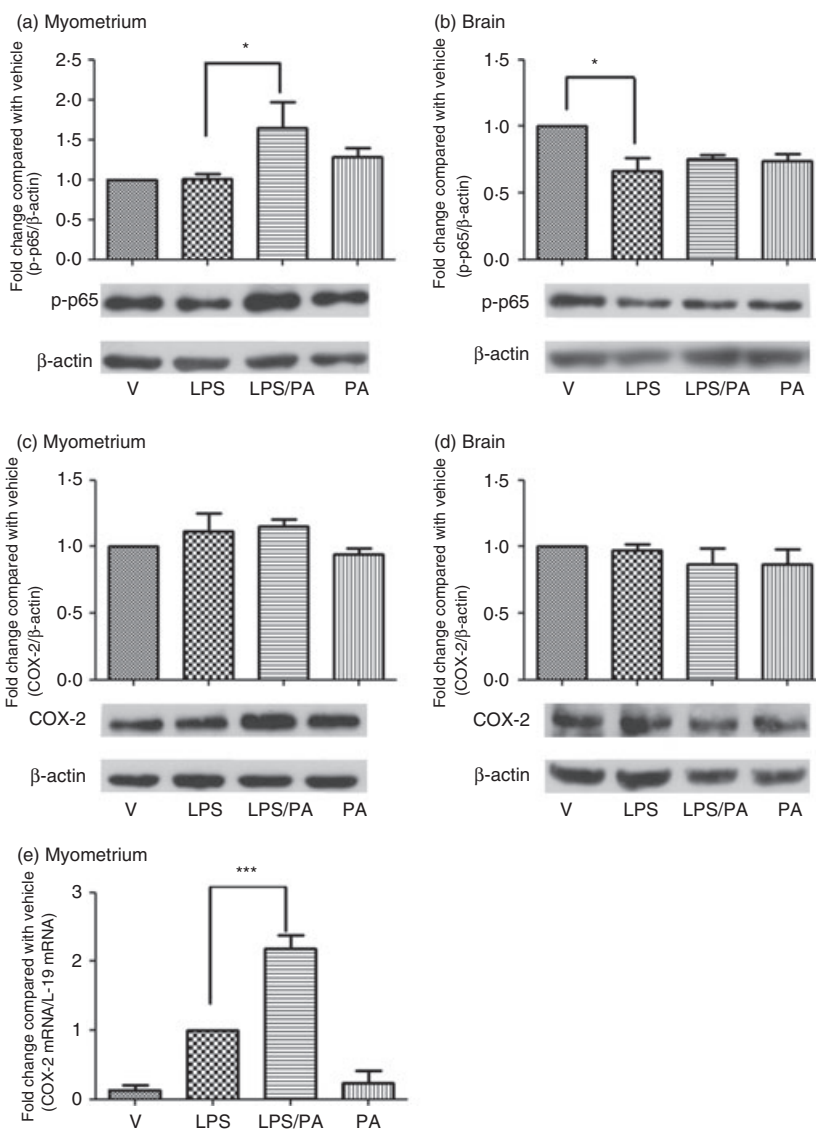


Figure 6. The effect of lipopolysaccharide (LPS) and Pyl A on myometrial and pup brain nuclear factor- κ B (NF- κ B) and cyclo-oxygenase 2 (COX 2). Dams were killed at 4.5 hr post intrauterine injection and tissue was harvested for protein analysis of phospho-p65 and COX-2 ($n = 3$). Co-injection of LPS and Pyl A increased myometrial phospho p65 (a), with no effect on pup brain phospho-p65. Phospho-p65 was decreased in brains of pups from dams treated with LPS alone (b). Representative phospho-p65 immunoblots are shown for each treatment group with B actin used as a loading control. No significant changes in COX-2 protein expression were seen in any treatment group in both myometrium and pup brain (c,d). However, an increase in messenger RNA of COX-2 was seen in LPS-treated and LPS/Pyl A-treated mice (e). For statistical analysis, one-way analysis of variance with Bonferroni's multiple comparison test was used; * $P < 0.05$, *** $P < 0.001$.

an increase in both mRNA and protein expression of IL-10, which did not achieve statistical significance (Fig. 8). These interleukins were not detectable in fetal brain samples (data not shown).

The effect of Pyl A on uterine contractility

To determine if Pyl A had a direct effect on uterine contractility, uteri were harvested from mice on E15–16, dissected and mounted on the myograph in the circular orientation. Pyl A inhibited myometrial contractility from a concentration of $10 \mu\text{M}$ ($P < 0.01$), with complete inhibition seen with $100 \mu\text{M}$ ($P < 0.001$) (Fig. 9a,b). The effect of Pyl A on longitudinal muscle was also examined by mounting the strips along the longitudinal orientation. Contractility was not maintained in the longitudinal orientation for the whole duration of the experiment in control strips to robustly examine the effect of Pyl A on

longitudinal muscle contractility. Despite this, the clear inhibition seen in the circular muscle was not evident in the longitudinal strips (data not shown). The inhibition of contractility in circular muscle was probably not CRTH2-mediated because other agonists, 15dPGJ_2 and $13,14\text{-dihydro-15-keto-prostaglandin D}_2$ (DK-PGD $_2$), did not have the same effect (Fig. 9c–f).

Discussion

The search for preventative therapies for both preterm birth and related neurological injury has largely focused upon anti-inflammatory strategies. It is generally accepted that parturition is a pro-inflammatory event, with preterm labour being associated with an exaggerated inflammatory response and infection. When women present in preterm labour, it is likely that inflammation precedes any clinical symptoms. We have previously reported that

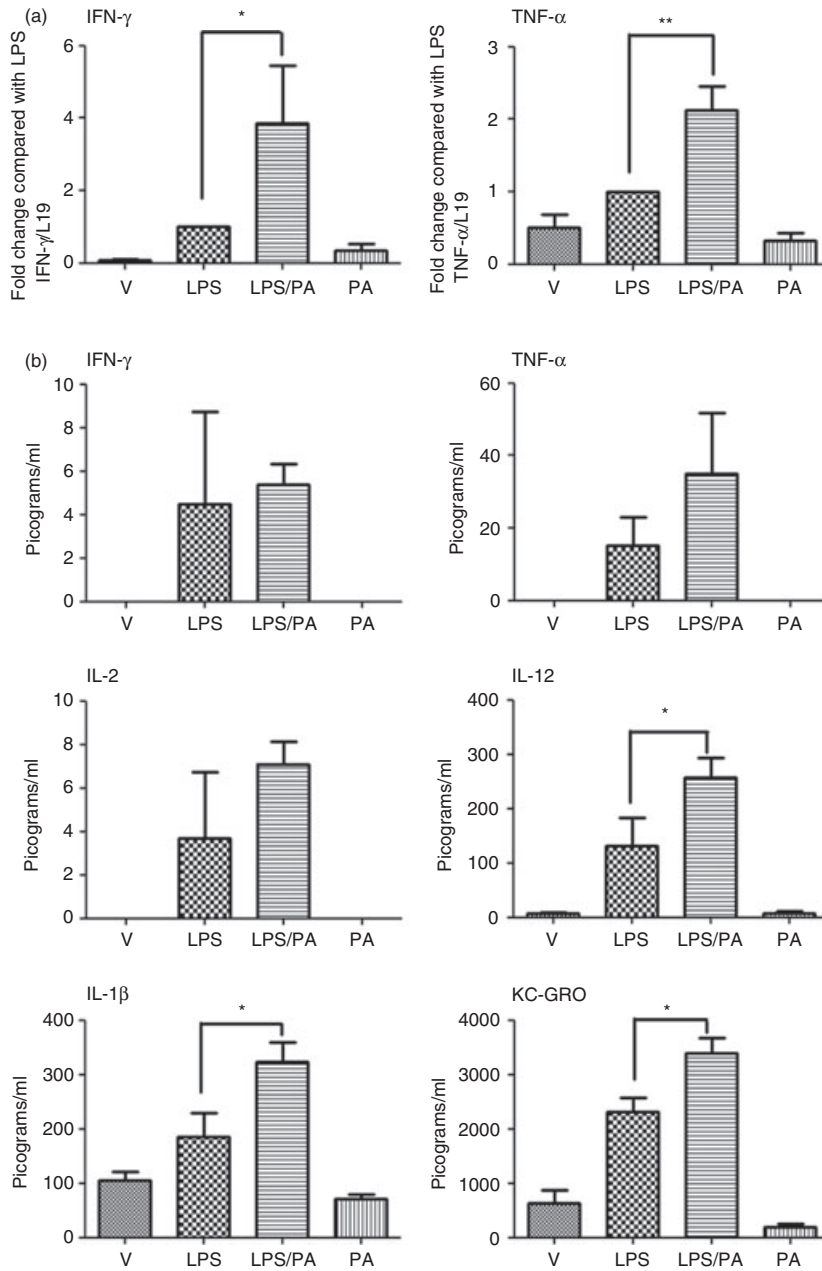


Figure 7. Effect of lipopolysaccharide (LPS) and Pyl A on the pro-inflammatory cytokines in the myometrium. Dams were killed at 4.5 hr post intrauterine injection of 20 μ g LPS/250 μ g Pyl A and mRNA and protein was extracted from the myometrium and analysed by PCR or ELISA ($n = 3$). With co-injection of LPS and Pyl A, interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) mRNA were significantly increased (a) as well as protein levels of interleukin-12 (IL-12), IL-1 β and KC-GRO (b). V = vehicle, PA = Pyl A. For statistical analysis, one-way analysis of variance with Bonferroni's multiple comparison's test was used; * $P < 0.05$, ** $P < 0.01$.

the anti-inflammatory cyclopentenone prostaglandin and CRTH2 agonist 15dPGJ₂ delays inflammation-induced preterm labour in the mouse and increases pup survival.¹³ In this study we have examined the potential for acute administration of a small molecule CRTH2 agonist to improve both maternal and fetal outcomes in LPS-induced murine preterm labour.

The CRTH2 agonist Pyl A was manufactured based on the L-888 607 compound from the Merck Frosst Centre for Therapeutic Research in which radioligand binding assays were used to demonstrate the affinity of the compound to the CRTH2 receptor.²⁵ The CRTH2 agonist activity of Pyl A was confirmed with a gold standard

experiment based on the work of Cossette, Monneret and Nagata, in which the CRTH2 agonists PGD₂, DK-PGD₂, indomethacin and 15dPGJ₂ cause up-regulation of CR3 (CD11b) in granulocytes.^{15,27,30-32} Pyl A caused a significant increase in the expression of CR3 (CD11b) in human eosinophils, which could be attenuated by pre-incubation with the CRTH2 antagonist GSKCRTH2X (Fig. 2), further confirming activity at the CRTH2 receptor. CR3 (CD11b) up-regulation via CRTH2 is believed to aid cell adhesion to the vascular wall for migration of cells from the blood into tissue at sites of inflammation.³³ The murine *CRTH2* gene was first cloned and characterized by Abe *et al.*³⁴ and shares 77% homology with the

Figure 8. Effect of lipopolysaccharide (LPS) and Pyl A on the anti-inflammatory cytokines in the myometrium. Dams were killed at 4.5 hr post intrauterine injection of 20 µg LPS/250 µg Pyl A and mRNA and protein was extracted from the myometrium and analysed by PCR or ELISA. No change in interleukin-4 (IL-4) mRNA expression was seen, and a non-significant increase in IL-10 was seen with co-administration of Pyl A and LPS (a). Although there was an increase in protein levels of IL-10 and IL-5 with co-injection of LPS and Pyl A, this did not reach statistical significance (b). V = Vehicle, PA = Pyl A.

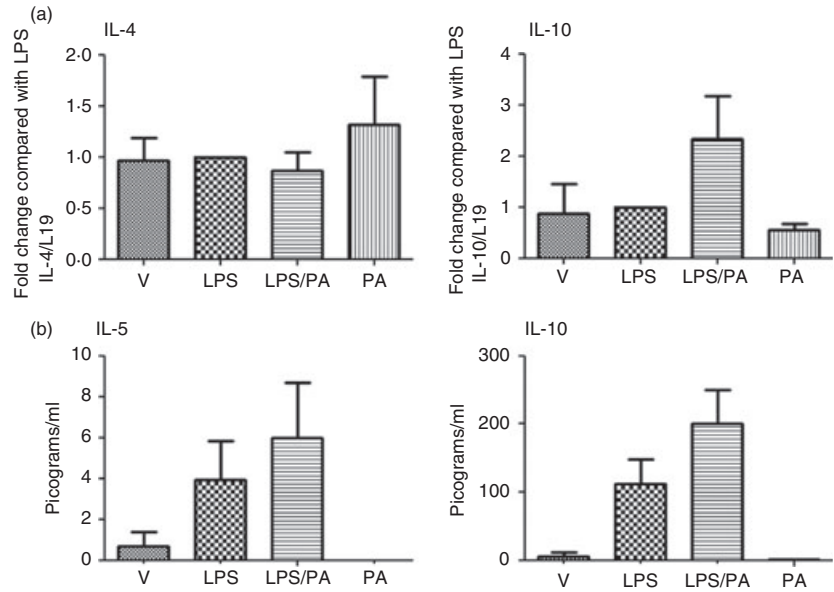
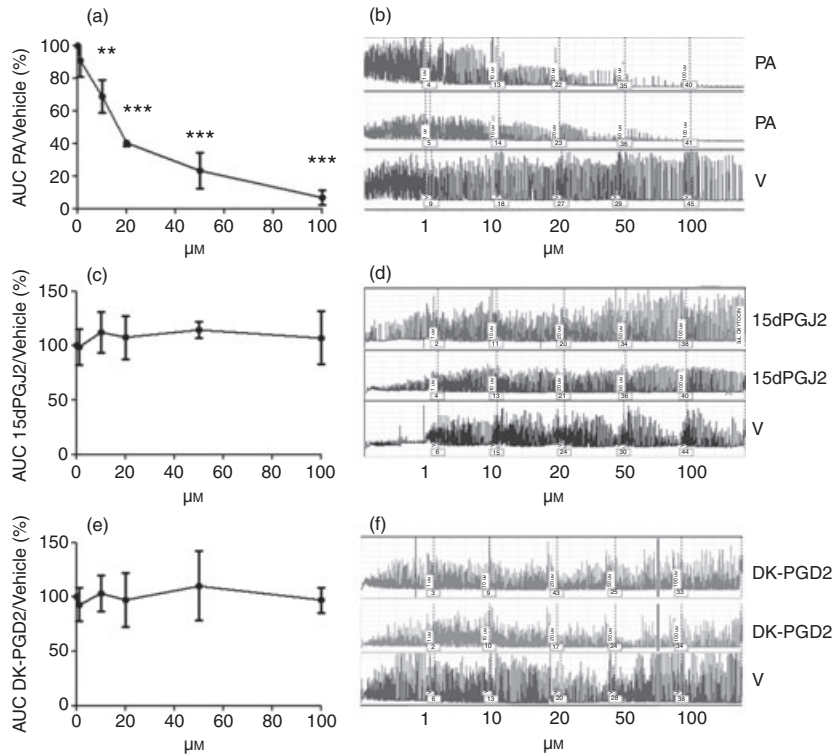


Figure 9. The effect of CRTH2 agonists on circular muscle contractility. CD1 mice were killed on E15–16 and myometrial tissue was harvested to mount on the myograph in the circular orientation ($n = 3$). A cumulative dose-response of CRTH2 agonists or vehicle was performed and the average area under the curve was determined. An inhibition in myometrial contractility was seen with Pyl A, but no effect with 15dPGJ₂ or DK-PGD₂. Graphs showing the effects of the CRTH2 agonists are shown (a,c,e), with representative traces of myometrial contractility (b,d,f). V = vehicle, PA = Pyl A. For statistical analysis, analysis of variance of repeated measures was used with Dunnett's multiple comparison test; ** $P < 0.01$, *** $P < 0.001$.



human CRTH2 receptor gene. The pharmacologies of the human and mouse CRTH2 receptors are virtually identical, and the receptors share 90% homology within the transmembrane domains.³⁵ The CRTH2 agonists PGD₂, DK-PGD₂, 15dPGJ₂ and indomethacin all show activity to the mouse CRTH2 receptor.^{36–39} 15dPGJ₂ binds to the mouse CRTH2 receptor with an affinity several orders of magnitude greater than that seen for peroxisome proliferator-activated receptor-γ.^{39,40} We detected CRTH2 mRNA in the mouse myometrium using the primers used by

Abe *et al.*,³⁴ (Fig. 1). There was no difference in mRNA expression between vehicle and Pyl A-treated or LPS-treated mice and LPS/Pyl A-treated mice. However, the degree of expression seen at the mRNA level suggests that CRTH2 is expressed in the myometrium. Determining if expression is seen on both myocytes and infiltrating leucocytes or leucocytes alone has not been possible because of the lack of available specific antibodies to murine CRTH2. Human studies have demonstrated mRNA expression in the myometrium, but flow cytometry

confirms the absence of the expressed protein in cultured myocytes.⁴¹ CRTH2 positive leucocytes are also detected in the endometrium and are likely to be recruited to decidua via PGD₂.^{42,43}

We have previously reported that the CRTH2 agonist 15dPGJ₂ delays LPS-induced preterm labour in the mouse, which is thought to be via NF- κ B inhibition in the myometrium.¹³ 15dPGJ₂ also inhibits NF- κ B in human cultured amniocytes and myocytes;¹² however, the mechanism by which NF- κ B inhibition is achieved is unclear. It was therefore hypothesized that Pyl A could reproduce the effects of 15dPGJ₂ of delaying preterm labour by diminishing the pro-inflammatory effect of LPS via NF- κ B inhibition. However, co-injection of LPS-treated mice with Pyl A was found to exacerbate time to preterm labour in a dose-dependent response (Fig. 4b). To establish the mechanism of Pyl A-augmented labour onset we first examined the effect of Pyl A and LPS on the pro-inflammatory and pro-labour transcription factor NF- κ B and its downstream targets. Treatment of animals with Pyl A alone increased NF- κ B activity in the myometrium, which was enhanced with co-administration of LPS (Fig. 6a). The inability of Pyl A to inhibit NF- κ B implies that CRTH2 is not involved in the mechanism of 15dPGJ₂-mediated inhibition. In support of this, we demonstrated that CRTH2 is not required for 15dPGJ₂-mediated inhibition of NF- κ B in human amniocytes, myocytes and lymphocytes.⁴¹

Surprisingly, myometrial COX-2 protein levels remained unchanged 4-5 hr post treatment in all groups. As preterm labour was typically induced following LPS/Pyl A treatment at 5-8 hr (SEM \pm 0.7) it was expected that any COX-2 up-regulation in the myometrium should have already been apparent by 4-5 hr post treatment. It is possible that COX-2 was already up-regulated before intrauterine injection in preparation for term labour, which is one limitation of using a model at E16. Progesterone withdrawal in the mouse occurs late E16 and so downstream activation of pro-labour genes is not likely to have been initiated in our model.⁴⁴ Consistent with this the majority of labour-associated proteins such as PGE₂, PGF_{2 α} , the oxytocin receptor and Connexin-43 are not significantly up-regulated until E18.^{45,46} We have shown, however, that COX-2 is suppressed in pregnancy and is up-regulated from E16, which was not increased further in term labour.⁴⁷ We further explored the possibility that, despite seeing no change at the protein level, COX-2 was still activated by LPS and LPS plus Pyl A. Messenger RNA was indeed increased in LPS-treated mice, and was further increased with co-injection of Pyl A (Fig. 6e). COX-2 requires peroxidases for activation and the endogenous peroxide tone of smooth muscle cells can be mimicked by nitration.⁴⁸ Previous studies have shown that peroxynitrite increases the activity of COX-2 with no alteration of COX-2 protein expression.^{49,50} Con-

sistent with our results, Aisemberg *et al.*⁵¹ demonstrated an increase in LPS-induced mRNA COX-2 with no effect at the protein level. It is plausible that this is a result of LPS-induced NO leading to the formation of peroxynitrite, which in turn, activates COX-2 without alteration of protein expression. Alternatively, it is also plausible that the nitrated form of COX-2 is not recognized by the COX-2 antibody.

Analysis of pup brain extracts collected from LPS-treated dams revealed a decrease in levels of phosphorylated p65 (ser 536). It is thought that this may reflect protein degradation induced by the pre-terminal state of the live pups (Fig. 6b). A significant increase in *in utero* fetal viability was achieved with Pyl A treatment (Fig. 5a) but this was not associated with altered NF- κ B activity. This also highlights the contrasting effects of Pyl A compared with the 15dPGJ₂ because we have previously shown that 15dPGJ₂ inhibits NF- κ B in the pup brain of dams treated with LPS.¹³ Co-injection of Pyl A and LPS led to an increase in the production of the pro-inflammatory cytokines TNF- α , IFN- γ , IL-12, IL-1 β and IL-8 (Fig. 7b). Pro-inflammatory stimuli such as LPS⁵² and the cytokines TNF- α ⁵³ and IL-1⁵⁴ have been shown to activate NF- κ B via the canonical pathway by phosphorylating serines, leading to I kappa B (I κ B) degradation and translocation of the NF- κ B complex into the nucleus, thereby activating gene expression of pro-inflammatory cytokines, which augments the pro-inflammatory response in a positive feed-forward loop.⁵ The assessed cytokines were also increased but to a lesser degree in the absence of NF- κ B activation with LPS treatment alone (Fig. 7a,b). It is therefore plausible that LPS and Pyl A co-administration activates a strong cytokine response, which then further induces NF- κ B activation via a feed-forward mechanism. Lipopolysaccharide induces a strong inflammatory response and leads to the recruitment of leucocytes.^{55,56} CRTH2 agonists also chemoattract CRTH2-positive leucocytes,^{19,57} including Th2 cells,¹⁹ eosinophils⁵⁸ and dendritic cells.³⁷ The increase in inflammatory cytokines seen with combined injection of both LPS and the CRTH2 agonist Pyl A may be as a result of the increase in infiltrating leucocytes rather than a direct effect on myocytes. Importantly, CRTH2 is also expressed on Th1 cells in the mouse, unlike the human, which is likely to have contributed to the unexpected pro-inflammatory response seen in the mouse.

Several murine studies with CRTH2 agonists/antagonists and the use of CRTH2 knock-out mice have shown a pro-inflammatory role for the CRTH2 receptor.^{36,38,59-63} The CRTH2 agonist DK-PGD₂ causes eosinophilia in mouse lung⁶³ and intra-peritoneal administration of DK-PGD₂ causes a two-fold induction of monocyte chemoattractant protein-1 and a 25-fold induction of macrophage inflammatory protein-2.³⁶ Furthermore, in a murine study of FITC-induced inflammation of the skin (a model of

contact hypersensitivity), a CRTH2 antagonist was found to significantly reduce the production of the pro-inflammatory cytokines TNF- α , IL-1 β and the chemokines macrophage inflammatory protein-2 and GRO- α .⁶⁴ However, no distinction between the Th1 or Th2 type cytokines being modulated was made. Similarly reduced levels of lung IFN- γ , IL-4 and IL-5 have been observed in a mouse model of airway inflammation upon administration of a CRTH2 antagonist.⁶²

Our finding of increased fetal viability with Pyl A in LPS-treated mice was surprising in view of the shortened time interval from injection to delivery. Although following spontaneous labour there were no surviving pups in the LPS and LPS/Pyl A treatment groups (Fig. 5b). We attribute this to the pups delivering preterm, based on unpublished data showing non-viability at E16 even in the absence of inflammation-induced preterm labour. The CRTH2 agonists indomethacin and PGD₂ lead to an increase in the production of the Th2 cytokines in human T cells *in vitro*,^{22,30} which in turn can antagonize the Th1 response. *In vitro* stimulation of Th2 cells by PGD₂ requires much higher concentrations to stimulate IL-10 production compared with IL-4, IL-5 and IL-13.^{1,22} We therefore examined the effect of Pyl A on the Th2-type anti-inflammatory cytokines in the myometrium (Fig. 8). Although no changes in levels of IL-4 were detected, an increase (non-significant) in IL-5 was observed (Fig. 8). Moreover, a non-significant increase in IL-10 mRNA and protein with LPS and Pyl A treatment was detected consistent with improved protection against LPS-induced fetal loss in mice⁶⁵ as well as the reduced rate of naturally occurring fetal loss in IL-10-deficient mice.²⁴

Although Pyl A led to a small increase in the pro-labour transcription factor NF- κ B and the pro-inflammatory cytokines, we did not see an increase in COX-2 protein expression. We therefore examined the direct effect of Pyl A on myometrial contractility *ex vivo*. Contrary to the expected uterotonic effect, Pyl A administration resulted in complete inhibition of circular muscle contractility (Fig. 9), but had no effect on longitudinal muscle. There is limited knowledge on the functional role of the individual muscle layers of the mouse uterus, the inner circular and outer longitudinal muscle, in pregnancy and parturition. In the myometrium of other species such as the pig and rat, it has been suggested that the function of the longitudinal muscle is to move luminal contents by contraction⁶⁶ and that tonic contraction of the circular muscle may be required for spacing and retention of embryos/fetuses.⁶⁷ Circular muscle cells have a higher spontaneous electrical activity than longitudinal muscle cells during rat pregnancy,⁶⁸ and weak high-frequency contractions in the circular muscle layer prevent movement of fetuses towards the cervix during pregnancy,⁶⁹ supporting its potential role in the maintenance of pregnancy. If circular muscle contraction is

necessary for retention of uterine contents, this would explain how inhibition of circular muscle contraction by Pyl A leads to preterm expulsion of the fetuses, as seen in this study. Consistent with this, relaxation of uterine tone is also believed to be important during human labour.⁷⁰ It is proposed that relaxation of the lower segment of the uterus, in conjunction with contractions of the fundal region, is required for the passage of the fetus through the birth canal.

Alternatively, relaxation of circular muscle may not be important in murine labour. Many rodent studies suggest that by term, the function of circular muscle becomes more similar to the longitudinal layer, and that contractility of both the circular and longitudinal muscle is required for labour.^{71–74} It is possible that despite the inhibitory effect on contractions seen with Pyl A *ex vivo*, that the overwhelming *in vivo* inflammatory effect was enough to overcome the tocolytic effect resulting in preterm labour. It is also plausible that the *in vivo* uterotonic effect of Pyl A occurs through indirect stimulation of immune cells and activation of the myometrium via an inflammatory response, rather than a direct action of Pyl A on myocytes. This is comparable to the indirect effect of LPS-induced labour, because addition of LPS to myometrial strips *ex vivo* does not lead to increased myometrial contractility.

The observed inhibition in myometrial contractility seen with Pyl A is likely to be through a CRTH2-independent mechanism as the other CRTH2 agonists 15dPGJ₂ and DK-PGD₂ did not show the same effect. At higher concentrations, Pyl A is able to bind to other prostanoid receptors with the rank of order of affinity as follows: CRTH2 > TP > EP3 > DP > EP4 > EP2 > FP > IP > EP1.²⁵ Since the TP/IP/EP3/EP1 receptors are considered to be excitatory and the EP2/EP4 and DP1 receptors relaxatory, we hypothesize that Pyl A may be having off-target effects on one of the latter mentioned receptors. The effect of DP1 agonists on murine contractility has been investigated previously by several groups. We have shown that the EP2 agonist, but not EP4 agonist, inhibits human myometrial contractility.⁷⁵ Stimulation of the EP2 and EP4 receptors leads to cAMP production via the G protein Gs leading to smooth muscle relaxation.⁷⁶ Hence the effect seen in our study is potentially a result of non-specific binding with the EP2 receptor.

Conclusion

This study presents evidence that the CRTH2 agonist Pyl A augments a pro-inflammatory response in LPS-induced preterm labour in the mouse. Pyl A shortened the time interval from intrauterine injection to preterm delivery via increased NF- κ B activity and increased production of pro-inflammatory cytokines. We also demonstrated a non-CRTH2-mediated inhibition of circular myometrial contractility *ex vivo*, which was likely to contribute to

rapid expulsion of the fetus. Despite increased fetal viability seen with Pyl A in LPS-treated dams, an overwhelming pro-inflammatory response was seen with the CRTH2 agonist in the mouse. This may be secondary to a functional CRTH2 receptor on murine Th1 cells, unlike in humans. We conclude that 15dPGJ₂-mediated inhibition of NF- κ B is not mediated via CRTH2. The CRTH2 agonist seems to augment inflammation-induced preterm birth, so CRTH2 is unlikely to be a suitable therapeutic target for the prevention of preterm labour and neonatal morbidity.

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Disclosures

The authors have no financial disclosures or competing interests.

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