Effects of cytokine-suppressive anti-inflammatory drugs on inflammatory activation in *ex vivo* human and ovine fetal membranes

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Abstract

Intrauterine infection and inflammation are responsible for the majority of early (<32 weeks) spontaneous preterm births (PTBs). Anti-inflammatory agents, delivered intra-amniotically together with antibiotics, may be an effective strategy for preventing PTB. In this study, the effects of four cytokine-suppressive anti-inflammatory drugs (CSAIDs: N-acetyl cysteine (NAC), SB239063, TPCA-1 and NEMO binding domain inhibitor (NBDI)) were assessed on human and ovine gestational membrane inflammation. Full-thickness membranes were collected from healthy, term, human placentas delivered by Caesarean section ($n=5$). Using a Transwell model, they were stimulated *ex vivo* with γ -irradiation-killed *Escherichia coli* applied to the amniotic face. Membranes from near-term, ovine placentas were stimulated *in utero* with lipopolysaccharide, *Ureaplasma parvum* or saline control and subjected to explant culture. The effects of treatment with CSAIDs or vehicle (1% DMSO) on accumulation of PGE₂ and cytokines (human interleukin 6 (IL6), IL10 and TNF α ; ovine IL8 (oIL8)) were assessed in conditioned media at various time points (3–20 h). In human membranes, the IKK β inhibitor TPCA-1 (7 μ M) and p38 MAPK inhibitor SB239063 (20 μ M) administered to the amniotic compartment were the most effective in inhibiting accumulation of cytokines and PGE₂ in the fetal compartment. NAC (10 mM) inhibited accumulation of PGE₂ and IL10 only; NBDI (10 μ M) had no significant effect. In addition to the fetal compartment, SB239063 also exerted consistent and significant inhibitory effects in the maternal compartment. TPCA-1 and SB239063 suppressed oIL8 production, while all CSAIDs tested suppressed ovine PGE₂ production. These results support the further investigation of intra-amniotically delivered CSAIDs for the prevention of inflammation-mediated PTB.

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Introduction

Preterm birth (PTB) remains a persistent obstetric challenge associated with significantly increased risk of neonatal mortality as well as short- and long-term morbidities (Goldenberg *et al.* 2008). The worldwide PTB rate is around 9.6%, with rates typically lower in developed countries (5–8%) and higher in developing nations (8–18%) (Lawn *et al.* 2010). In addition to its impact on individuals and their families, PTB carries a substantial economic cost, estimated to be ~\$26 billion annually in the USA in 2005 (Behrman & Stith Butler 2007). Intrauterine infection and associated inflammation (most frequently diagnosed as presence of histological chorioamnionitis) have been identified as a cause of 30–40% of all spontaneous PTB (sPTB). Up to 70% of very early sPTBs (<32 weeks gestation) are due to intrauterine infection–inflammation (Goldenberg *et al.* 2008).

Prophylactic antibiotic therapy has been extensively studied in the context of PTB prevention, with mixed results. While some studies have shown that administration of antibiotics (e.g. clindamycin) to high-risk women early in pregnancy (<20 weeks gestation) can have positive benefits in terms of reduced PTB rates and improved perinatal outcomes (Lamont *et al.* 2011), the majority of trials have failed to show significant benefits (Barros *et al.* 2010) and in some studies have even been shown to be harmful. The reasons for this are several fold and include issues related to participant selection and antibiotic efficacy, tissue biodistribution and microbial resistance (Keelan 2011). In addition, bactericidal antibiotics cause bacterial lysis and release of endotoxins, further activating the innate immune system and promoting the release of prostaglandins that may actually stimulate the onset of labour (Dofferhoff *et al.* 1991, Hurley 1995, Holzheimer 2001).

We and others have proposed that a combined anti-inflammatory/antibiotic approach may be more effective than antibiotics alone in treating intrauterine infection–inflammation, prolonging pregnancy and preventing fetal exposure to an inflammatory environment (Keelan 2011, Grigsby *et al.* 2012). Our present focus is on the intra-amniotic administration of anti-inflammatory/antimicrobial agents in order to maximise therapeutic efficacy at the site of infection/inflammation, while minimising the risks of undesirable side effects through the reduction of unintended maternal or fetal exposure. Most of the literature on anti-inflammatory drugs in PTB has focussed on non-steroidal anti-inflammatory drugs (NSAIDs) – prostaglandin synthesis inhibitors that have widespread applications but which have been associated with significant fetal side effects (Kaplan *et al.* 1994, Nakhai-Pour *et al.* 2011). On the other hand, cytokine-suppressive anti-inflammatory drugs (CSAIDs) work by interfering with inflammatory signalling cascades and are therefore able to specifically block infection-mediated inflammation without some of the deleterious side effects of NSAIDs (Lee *et al.* 1989, Keelan 2011). CSAIDs have been shown to block inflammation in a variety of animal models of chronic inflammation (Underwood *et al.* 2000, Ward *et al.* 2001, Buhimschi *et al.* 2003, Jimi *et al.* 2004, di Meglio *et al.* 2005) as well as in human fetal membranes (Lappas *et al.* 2003, De Silva *et al.* 2010).

In this study, we evaluated four CSAIDs that target two key signalling pathways known to be involved in inflammatory activation of fetal membranes: nuclear factor- κ B (NF- κ B; Lindstrom & Bennett 2005) and p38 MAPK (Lappas *et al.* 2007). The CSAIDs were as follows: i) NEMO binding domain inhibitor (NBDI), ii) *N*-acetyl cysteine (NAC), iii) TPCA-1 ((5-(*p*-fluorophenyl)-2-ureido) thiophene-3-carboxamide) and iv) SB239063 (*trans*-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-(methoxy)pyrimidin-4-yl)imidazole). An intra-amniotic model of drug delivery was employed, as this approach allows for the targeting of gestational membranes and tissues (the key sites with respect to intra-amniotic infection-driven PTB) with minimal risk of unintended maternal immune modulation.

This study aimed to assess and compare the anti-inflammatory efficacies of the four chosen CSAIDs on human and ovine gestational membranes using *ex vivo* models of intra-amniotic drug administration to assess their ability to inhibit inflammatory activation in both the amniotic and decidual faces of the gestational membranes.

Subjects and methods

CSAIDs

The concentrations of CSAIDs were as follows: NAC (Enzo Life Sciences, New York, NY, USA) 10 mM, NBDI (China Peptides, Shanghai, China) 10 μ M, TPCA-1 (Merck Millipore, Darmstadt,

Germany) 7 μ M and SB239063 (Alexis Biochemicals, Lausen, Switzerland) 20 μ M. Doses of CSAIDs were based on pilot studies or published data on *in vitro* efficacy (Underwood *et al.* 2000, Barone *et al.* 2001, Ward *et al.* 2001, Ju *et al.* 2002, Lappas *et al.* 2003, Jimi *et al.* 2004, di Meglio *et al.* 2005, Tas *et al.* 2006, Shahin *et al.* 2009, De Silva *et al.* 2010, Grassia *et al.* 2010).

Human membrane Transwell model

Full-thickness gestational membranes were collected from healthy, human, term placentas (38–40 weeks of gestation) delivered by Caesarean section ($n=5$) with the approval of the local institutional Human Research Ethics Committee. Membranes were secured over 27 mm Transwell inserts (Corning, Inc., Lindfield, NSW, Australia) and placed in six-well culture plates containing serum-free culture media (DMEM/Ham's Nutrient Mixture F-12, phenol red-free, supplemented with 15 mM HEPES, pH 7.3 (Sigma–Aldrich Co.), 0.5% endotoxin/fatty acid-free BSA (Bovogen Biologicals Pty Ltd, East Keilor, VIC, Australia) and 4 μ g/ml azithromycin (Pfizer, New York, NY, USA)). The maternal/decidual compartment contained 3 ml media, while the inner/amniotic compartment contained 2.5 ml. γ -irradiation-killed *Escherichia coli* (10 μ g/ml) and fluorescent Spherobeads (4060 nm, 0.1 mg/ml; Spherotech, Inc., Lake Forrest, IL, USA) were added to the inner compartment, followed by CSAIDs in 1% DMSO or vehicle (1% DMSO control) at $t=0$ h. Membranes were incubated for 20 h at 37 °C in 5% CO₂/95% air. Samples of conditioned media (100 μ l) were taken from the fetal and maternal compartments at 0, 3 and 9 h and a final 1 ml sample was taken at 20 h. Structural integrity of the membranes was monitored by the passage of Spherobeads between inner/amniotic compartment and maternal/decidual compartments. Spherobead concentrations were measured in the fetal and maternal compartments by fluorescence using an FLx 800 plate fluorometer (BioTek Instruments, Inc., Winooski, VT, USA) at excitation 585/10 nm and emission 620/15 nm. Analysis of samples from both compartments of the Transwells showed that all membranes were intact with >99% of Spherobeads retained within the fetal compartments and no significant fluorescence detected in the maternal compartments of any of the Transwells.

Ovine membrane explant studies

Animal studies were performed on pregnant Merino sheep (*Ovis aries*) in Western Australia with the approval of the University of Western Australia's Animal Ethics Committee (RA/3/100/1098). The sheep in this study received intra-amniotic injections at 117 ± 2 days gestational age (GA) of saline (2 ml, $n=2$), lipopolysaccharide (LPS; O55:B5; Sigma–Aldrich; 10 mg in 2 ml saline, $n=4$) or *Ureaplasma parvum* serovar 3 (10^7 colour change units in 2 ml saline, $n=4$) 7 days prior to delivery. All fetuses were surgically delivered at 124 ± 2 days GA (term=150 days) for necropsy. Fetal membranes were excised at this time and transported to the laboratory in media for explant culture. Explants were prepared from each set of membranes (8 mm discs), with three discs placed per well in 12-well plates and incubated in serum-free culture media at 37 °C/5% CO₂/95% air. Treatment with the

CSAIDs or vehicle (DMSO, 1%) was carried out for 14 h before the explants were removed, the media were stored at -80°C for later analysis and the tissues were air-dried overnight and weighed for normalisation.

Measurement of cytokine and PGE₂ concentrations

Accumulation of cytokines (human interleukin 10 (IL10), IL6 and TNF α ; ovine IL8 (oIL8)) and PGE₂ was measured in conditioned fetal and maternal media for the human Transwell study and from explant conditioned media for the ovine studies. Human IL10 and TNF α were measured by multiplex assay (Merck Millipore, Darmstadt, Germany) on a MAGPIX Instrument (Luminex Corp., Austin, TX, USA) as per the manufacturer's instructions. Human IL6 was measured using an ELISA Development Kit (PeproTech, Rocky Hill, NJ, USA) according to the recommended protocol. PGE₂ was measured by prostaglandin E₂ EIA Kit – monoclonal (Cayman Chemical Company, Ann Arbor, MI, USA) as per the manufacturer's instructions. oIL8 was measured by in-house ELISA calibrated against recombinant oIL8 from Protein Express, Inc. (Cincinnati, OH, USA) using a mouse anti-sheep IL8 monoclonal capture antibody (MCA1660: 5 $\mu\text{g}/\text{ml}$ overnight) and a rabbit anti-sheep IL8 polyclonal antibody (AHP425: 1:1000 2 h) from AbD Serotec (Raleigh, NC, USA). Detection and quantitation involved an anti-rabbit IgG–HRPO conjugate (1:1000 1 h) and TMB substrate. The limits of detection of the IL10, TNF α , IL6, PGE₂ and oIL8 assays were <3.2 , <3.2 , 100, 7 and 33 pg/ml respectively. Media samples were diluted 1:10 for the IL6 assay, 1:5 for the PGE₂ assay, 1:2 for the IL8 assay and were undiluted for the IL10 and TNF α assays.

Statistical analysis

To adjust for variable baseline expression between membranes from different placentas, the concentrations of cytokines and PGE₂ within the conditioned media from each Transwell were expressed as a percentage of the sum of concentrations from all six Transwells from each set of membranes. The production data from the sheep explants were similarly normalised prior to statistical analysis. Data are shown as median $\pm$ interquartile range (IQR) or mean $\pm$ S.E.M. Unless stated otherwise, all statistical significance was assessed by one-way ANOVA followed by Dunnett's *t*-test *post-hoc* analyses (Prism, GraphPad Software, Inc., La Jolla, CA, USA). Non-parametric data were log transformed prior to analysis. A *P* value of <0.05 was considered significant. For analysis of basal and stimulated cytokine production rates in human membranes, significance was assessed by Wilcoxon matched pairs test, both at each individual time point and overall.

Results

Cytokine and prostaglandin production by stimulated human fetal membranes in the Transwell perfusion model

Figure 1 shows the baseline production of cytokines and PGE₂ over time (3, 9 and 20 h) in vehicle (DMSO) or

Figure 1 Time-dependent changes in accumulation of (A) PGE₂, (B) TNF α and (C) IL10 in the conditioned media from the maternal and fetal compartments of human fetal membranes in the Transwell model following exposure to vehicle (basal) or 10 $\mu\text{g}/\text{ml}$ γ -irradiation killed *Escherichia coli* (stimulated) at the amniotic face. Data shown are concentration (pg/ml), mean $\pm$ S.E.M. ($n=5$ sets of membranes). * $P<0.05$ and ^ $P<0.001$ basal vs stimulated by Wilcoxon matched pairs test.

E. coli-stimulated human Transwells. Basal PGE₂ accumulation in the fetal compartment increased modestly from 3 to 9 h, then declined at 20 h (Fig. 1A); mean concentrations at 9 h were ~ 700 pg/ml. Basal PGE₂ levels were a little higher in the maternal compartment, peaking at ~ 1000 pg/ml at 9 h before declining by 20 h. With bacterial stimulation, however, levels in the fetal compartment rose markedly at 3 h to >1700 pg/ml and then declined thereafter, whereas in the maternal compartment no evidence of stimulation was observed. The effect of stimulation in the fetal compartment was significant at 20 h ($P<0.05$) and over all time points ($P<0.001$). TNF α accumulation in the fetal compartment under basal conditions also peaked at the 9-h incubation period, reaching ~ 1300 pg/ml (Fig. 1B). Similarly, maternal basal TNF α concentrations also peaked at 9 h (~ 2900 pg/ml), then declined to ~ 1000 pg/ml at 20 h. With bacterial stimulation, concentrations of TNF α in the fetal compartment were

significantly (two- to threefold) elevated at 3 and 20 h ($P<0.05$; overall significance: $P<0.001$), with a significant stimulation also seen in the maternal compartment ($P<0.05$ overall). Fetal IL10 levels were low or undetectable at 3 h, but rose to concentrations of ~ 300 pg/ml at 9 h before declining at 20 h (Fig. 1C). Maternal basal IL10 levels were significantly higher than fetal levels at 3 h ($P<0.01$) and peaked at 1100 pg/ml at 9 h, after which they progressively declined to 20 h. Stimulation with *E. coli* failed to increase IL10 levels in either compartment.

Anti-inflammatory effects of CSAIDs on human fetal membranes

Treatment of *E. coli*-stimulated human gestational membranes at the amniotic face with NBDI had no significant effects on PGE₂ accumulation in either compartment, although at the 9-h time point median maternal PGE₂ levels were reduced by $\sim 50\%$ (Fig. 2A). Treatment with NAC resulted in a non-significant 60% reduction in PGE₂ accumulation relative to DMSO controls in the fetal compartment at 9 h and a smaller ($\sim 35\%$) reduction in the maternal compartment at 20 h (Fig. 2A); TPCA-1 resulted in significant ($\sim 70\%$; $P<0.05$) suppression of PGE₂ accumulation in the fetal (but not maternal) compartment at 3, 9 and 20 h post treatment (Fig. 2A). SB239063 also significantly inhibited PGE₂ accumulation at all time points in the fetal compartment (80–85%; $P<0.05$), but unlike TPCA-1, it was also able to significantly reduce PGE₂ levels in the maternal compartment (~ 70 and 87% at 9 and 20 h; $P<0.05$ and <0.001 respectively).

Neither NBDI nor NAC significantly affected TNF α levels at any time point (Fig. 2B). TNF α accumulation in the fetal compartment was, however, markedly reduced by both TPCA-1 and SB239063, with significant reductions observed at 3 h ($P<0.01$), becoming more evident at 9 and 20 h ($P<0.001$). TPCA-1 significantly reduced maternal TNF α accumulation by $\sim 75\%$ at 9 h ($P<0.05$). SB239063 was again the most effective anti-inflammatory agent in the maternal compartment with significant inhibitions of ~ 95 and $\sim 62\%$ seen at 9 and 20 h respectively ($P<0.001$).

NBDI had no effect on IL10 production in either compartment; however, IL10 accumulation was inhibited by the other CSAIDs at the 9 and 20 h time points (Fig. 2C). Within the fetal compartment, NAC, TPCA-1 and SB239063 resulted in significant reductions in IL10 production with effects at 20 h in the region of 85–92% ($P<0.001$). The same three CSAIDs reduced IL10 accumulation in the maternal compartment, but the level of inhibition did not reach statistical significance due to large variability in the vehicle controls. The inhibitory effect of the anti-inflammatory agents tended to increase with time, although this trend was not statistically significant. The effect was most apparent for IL10 (Fig. 2C).

Inhibition of IL6 accumulation was assessed at 20 h only, due to insufficient media at the earlier time points. Once more, NBDI failed to exert significant effects. There was again a trend towards inhibition by NAC and TPCA-1 in the fetal compartment, although the degree of inhibition was more modest than that seen for the other cytokines (38–62%; Fig. 2D). SB239063 was the most effective anti-inflammatory agent in both compartments and significantly inhibited IL6 accumulation at the 20-h time point ($P<0.05$).

Anti-inflammatory effects of CSAIDs on ovine fetal membrane explants

The efficacies of the four CSAIDs were evaluated in full-thickness gestational membranes from near-term sheep. Explants were employed for the ovine studies as attempts to replicate the human Transwell study with ovine membranes were not successful. CSAID dosages and incubation times were based on the results from the human studies. Samples were initially assayed for oIL1 β , IL8, IL10, TNF α , MCP-1 and PGE₂; however, only concentrations of IL8 and PGE₂ were above the detection limits of the assays employed and generated meaningful data.

Median PGE₂ concentration in media from full-thickness gestational membrane explants from saline-treated sheep was 9.1 pg/mg tissue at 14 h, while median oIL8 levels were 53.0 pg/mg tissue. Membranes from sheep stimulated with LPS or *U. parvum* exhibited modest and variable increases in production of oIL8 and PGE₂ that did not reach statistical significance compared with saline-treated controls. Therefore, the data from all the three groups ($n=9$ sets of membranes) were analysed collectively.

In contrast to the human study, NBDI was as effective as the other CSAIDs at inhibiting PGE₂ accumulation in ovine gestational membranes. PGE₂ accumulation was significantly inhibited (65–71%; $P<0.01$) by all four CSAIDs compared with the DMSO vehicle-treated explants (Fig. 3A). However, NBDI and NAC had no effect on oIL8 levels, and the effects of NAC treatment on oIL8 levels were particularly variable. TPCA-1 significantly reduced oIL8 accumulation by 80% ($P<0.01$), while SB239063 significantly reduced oIL8 levels by $\sim 60\%$ ($P<0.01$) (Fig. 3B).

Discussion

PTB remains a major obstetric issue throughout the world and is associated with significant perinatal morbidity and mortality and lifelong health and economic consequences. In 2010, 14.9 million preterm deliveries occurred worldwide, from which over 1 million infants died as a result of their prematurity (Blencowe *et al.* 2012). Despite decades of research on PTB aetiologies, few therapeutic options are available to women at risk of delivering preterm. Here, we have

Figure 2 Efficacy of CSAIDs on *Escherichia coli* stimulated (A) PGE₂, (B) TNF α , (C) IL10 and (D) IL6 production by human full-thickness fetal membranes in an ex vivo Transwell perfusion model at 3-, 9- and 20-h culture. Data are median $\pm$ IQR from $n=5$ placentas, normalised as a percentage of total analyte production per set of experiments. * $P<0.05$, # $P<0.01$ and ^ $P<0.001$ relative to vehicle (DMSO) control. Significance was assessed by two-tailed ANOVA after log-transformation of data.

investigated the *ex vivo* efficacy of a number of anti-inflammatory agents based on the hypothesis that intra-amniotic CSAID administration can provide a pharmacological strategy for the prevention of infection/

inflammation-mediated PTB. The CSAIDs selected for this study were as follows: i) NBDI, a cell-permeable peptide that spans the NF- κ B essential modifier (NEMO) binding domain sequence (Madge & May 2009), which

Figure 3 Effects of CSAIDs on (A) PGE₂ and (B) IL8 accumulation in ovine fetal membranes ($n=9$) over 14 h in explant culture. Data are expressed as a percentage of total accumulation for each set of explants (median $\pm$ IQR). ** $P < 0.01$ relative to vehicle control (DMSO). Significance was assessed by two-tailed ANOVA after log-transformation of data.

has been shown to block inflammation effectively in *in vivo* animal models (Jimi *et al.* 2004, di Meglio *et al.* 2005, Tas *et al.* 2006, Grassia *et al.* 2010); ii) NAC, a powerful antioxidant and free radical scavenger that has been shown in a randomised controlled trial to reduce the rate of PTB when taken orally in women with a history of PTB and in whom bacterial vaginosis has recently been treated (Shahin *et al.* 2009); iii) TPCA-1, a selective IKK β inhibitor (Podolin *et al.* 2005, Kondo *et al.* 2008) that is effective at inhibiting inflammation *in vitro* (Podolin *et al.* 2005, Sachse *et al.* 2011) and *in vivo* (Birrell *et al.* 2006) and iv) SB239063, a selective, potent and cell-permeable p38 MAPK inhibitor that has previously been shown to suppress inflammation *in vivo* (Underwood *et al.* 2000, Barone *et al.* 2001, Ward *et al.* 2001, Ju *et al.* 2002). They were studied in a human Transwell system to model the structural characteristics of intact gestational membranes and allow the assessment of efficacy of intra-amniotic anti-inflammatory drug delivery at both the maternal and fetal faces of the membranes. In parallel, *in vivo* stimulated ovine fetal membranes were also exposed to the CSAIDs to allow

a comparison of efficacy between ovine and human tissues. DMSO vehicle was employed as previous studies in our laboratory have indicated that this solvent does not significantly alter inflammatory cytokine production by gestational tissues.

While all the four CSAIDs showed some degree of efficacy in both models, two were clearly superior: the IKK β inhibitor TPCA1 and the p38 MAPK inhibitor SB239063. At the concentrations employed, both these compounds induced profound inhibitory effects on cytokine and prostaglandin accumulation in the fetal compartment of the Transwell model, with the MAPK inhibitor exerting more modest effects in the maternal compartment. The same degree of inhibition by these two CSAIDs was also seen in the ovine explant model, regardless of mode of stimulation. These findings support our hypothesis and provide rationale for the further investigation of these compounds in human gestational tissues derived from spontaneous preterm deliveries.

The central importance of NF- κ B activation in the regulation of inflammatory gene expression is well recognised. We have previously shown that 5–7 μ M TPCA-1 achieved $\sim 90\%$ suppression of pro-inflammatory cytokine production and blocks nuclear translocation of p65/RelA in LPS-stimulated choriodecidual cells (De Silva *et al.* 2010). Until now, no studies have examined the effect of TPCA-1 in full-thickness gestational membranes, although it has been shown to be an effective inhibitor of the NF- κ B pathway in a variety of other inflammatory models (Podolin *et al.* 2005, Birrell *et al.* 2006, Kondo *et al.* 2008, Du *et al.* 2012). Interestingly, in this study, the actions of TPCA-1 were primarily restricted to the fetal (amniotic) compartment. This may reflect a lack of ability to penetrate the membrane barrier, restricting its actions to the amniotic epithelium, or may indicate that a higher dose is required to more completely block the transmembrane inflammatory signalling cascades. TPCA-1 also reduced PGE₂ and IL8 production from ovine fetal membranes, confirming its effectiveness as an IKK β inhibitor in the ovine species.

By contrast, the p38 MAPK inhibitor SB239063 was much more effective at inhibiting cytokine and prostaglandin accumulation at the maternal face, suggesting that it is either considerably more membrane permeable or has more profound effects on initial inflammatory signalling pathways. Its similar potency to TPCA-1 in the ovine explant model would argue against the latter hypothesis. To date, no studies have characterised the expression and activity patterns of MAPKs during inflammation in human gestational membranes, although MAPKs are known to respond to infectious stimuli and regulate the production of pro-inflammatory cytokines (Underwood *et al.* 2000, Barone *et al.* 2001, Ward *et al.* 2001, Ju *et al.* 2002, Shoji *et al.* 2007). Regardless, our results highlighted SB239063 as a potentially effective anti-inflammatory agent useful for preventing inflammation-driven PTB.

At the concentration used (10 μ M), NBDI was unable to inhibit production of any of the measured inflammatory markers in either compartment of the human Transwell model. We had selected a dose of 10 μ M of NBDI as this peptide has been successfully used at 0.1–1 μ M in a study of injury-induced inflammation in rats (Grassia *et al.* 2010). Surprisingly, it was notably more effective in the ovine explants at suppressing PGE₂ accumulation. It remains to be determined whether a species difference in binding affinity or a relative insensitivity of the amnion membrane might explain these observations.

NAC, which exerts its effects through dampening of oxygen free radical reactions, has been shown to suppress NF- κ B DNA binding activity in all the three layers of gestational membranes at ≥ 10 mM (Lappas *et al.* 2003). In our study, NAC (10 mM) was not a particularly effective inhibitor of fetal membrane cytokine production but did appear to reduce PGE₂ accumulation in the fetal side of the human Transwells. It also significantly reduced PGE₂ levels in conditioned media from sheep membrane explants. NAC can directly inhibit prostaglandin biosynthesis via inhibition of the production of PGH₂ by cyclooxygenases (De Flora *et al.* 2001), a reaction that involves a free radical step (Rouzer & Marnett 2009), so its effects on prostaglandin inhibition are consistent with expectations. These findings add some weight to the evidence that NAC might be an effective anti-inflammatory agent within the pregnant uterus (Lappas *et al.* 2003) and may be useful at preventing sPTB in some pregnancies (Shahin *et al.* 2009, Awad *et al.* 2011). The effectiveness of intra-amniotic delivery of NAC *in vivo* has not yet been explored.

The ovine membrane explant model used in this study employed membranes exposed *in vivo* to saline, LPS or *U. parvum*. This model has been developed over many years and is now extensively employed in obstetrics research (Kallapur *et al.* 2001, Moss *et al.* 2003, 2005). Unexpectedly, we did not observe a consistent and significant difference in PGE₂ or IL8 production from control or stimulated membranes, although mean levels of both mediators were two- to threefold higher in the stimulated membranes compared with controls. The inter-animal variability might have been due to regional differences in levels of activation of membranes. Owing to the lack of significance, the data from all membranes were combined and hence we are unable to make conclusions regarding the efficacy of the CSAIDs with respect to different stimuli.

In conclusion, the results presented in this study identified TPCA-1 and SB239063 as CSAIDs of promise for pharmacological prevention of intra-amniotic inflammation. Further *in vivo* studies are justified to explore their ability to ameliorate the negative effects of intrauterine infection-driven inflammation. In combination with an effective antibiotic regimen, CSAIDs administered intra-amniotically may have significant clinical benefits in treating pregnancies at high risk of sPTB due to intrauterine infection–inflammation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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