Sulfasalazine augments a pro-inflammatory response in interleukin-1β-stimulated amniocytes and myocytes

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Summary
Preterm birth occurs in 10% of pregnancies and is a major cause of neonatal morbidity and mortality. The majority of cases of early preterm labour are associated with infection/inflammation, which places the fetal central nervous system at risk. Targeting immune activation is therefore an appealing therapeutic strategy for the prevention of preterm labour and neonatal brain injury. The expression of many labour-associated and inflammatory-response genes is controlled by the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which makes them therapeutic targets of interest. Sulfasalazine (SASP) has been shown to inhibit NF-κB and reduce lipopolysaccharide-induced cytokine concentrations in fetal membrane explants and reduce the rate of Escherichia coli-induced preterm labour in mice. Its effects upon AP-1 in the context of pregnancy are unknown. In this study the effect of SASP on interleukin-1β (IL-1β)-induced NF-κB and AP-1 activity, cytokine production and cyclo-oxygenase-2 (COX-2) expression was examined in amniocytes and myocytes. A supra-therapeutic concentration (5 mM) was required to inhibit IL-1β-induced NF-κB (P < 0.001) in amniocytes and IL-1β-induced NF-κB (P < 0.01), AP-1 (P < 0.01) and COX-2 (P < 0.05) in myocytes. Despite inhibiting IL-1β-induced cytokines, a basal increase in IL-6 (P < 0.01), IL-8 (P < 0.0001) and tumour necrosis factor-α (TNF-α) (P < 0.001) was seen with 5 mM SASP in amniocytes, and significant cytotoxic effects were seen in myocytes. The therapeutic concentration of 0.015 mM had no inhibitory effects on pro-inflammatory mediators, but led to an augmented response to IL-1β-induced IL-6 (P < 0.01), IL-8 (P < 0.05) and TNF-α (P < 0.05) in amniocytes and IL-8 (P < 0.05) in myocytes. SASP is therefore an unlikely therapeutic candidate for the prevention of inflammation-induced preterm labour.

Keywords: activator of protein-1; amniocytes; interleukins; myocytes; nuclear factor-κB; sulfasalazine.

Introduction
Preterm birth rates are increasing globally. Worldwide, 1 in 10 babies are being born early. Despite increased efforts to understand the pathophysiology of preterm labour, this has not yet translated to a reduction in its incidence. In response, the World Health Organization has recently begun a global campaign to halve the number of deaths due to preterm birth by 2025. Although the triggers for the onset of preterm labour are not fully understood, a strong association exists between infection/inflammation and the premature activation of key molecular pathways required for the onset of labour. Despite infection being demonstrated in 40–80% of preterm

Abbreviations: ANOVA, analysis of variance; 5-ASA, 5-aminosalicylic acid; AP-1, activator protein-1; COX-2, cyclo-oxygenase-2; DMEM, Dulbecco’s modified Eagle’s medium; IKKα, inhibitor of nuclear factor-κB kinase α; IL, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; SASP, sulfasalazine; TNF-α, tumour necrosis factor-α
births. Antibiotics have not been shown to be effective in its prevention. There is mounting evidence that inflammation in the absence of infection is sufficient not only to activate molecular pathways necessary for the onset of labour, but also to contribute independently to poor outcome in neonates born preterm. It therefore follows that the use of anti-inflammatory therapies for the treatment of preterm birth should be explored.

Sulfasalazine (SASP) is an anti-inflammatory synthetic drug introduced in the 1930s, which combines the antibiotic sulfapyridine and 5-aminosalicylic acid (5-ASA). It is widely used for the treatment of inflammatory bowel disease and rheumatoid arthritis, and has a good safety record in pregnancy. A large cohort study reported almost a 50% reduction in preterm birth in women with Crohn’s disease treated with SASP or 5-ASA compared with untreated disease-matched controls.

Additionally, subcutaneous injection of SASP reduces preterm delivery rates in Escherichia coli-induced preterm labour in the mouse. The anti-inflammatory properties of SASP are thought to be attributable at least in part to its ability to inhibit nuclear factor-kB (NF-kB). Indeed, at supra-therapeutic concentrations SASP has been shown to significantly inhibit lipopolysaccharide (LPS)-induced NF-kB-binding activity and LPS-induced production of interleukin-6 (IL-6), IL-8 and tumour necrosis factor (TNF-α) in placental and fetal membrane explants.

The transcription factor NF-kB is a key regulator of both contraction-associated genes and pro-inflammatory cytokines. Nuclear factor-kB activates and is activated by the pro-inflammatory cytokines IL-8, IL-1β, TNF-α and IL-6, therefore, in the presence of inflammation this leads to a feed-forward effect. There is good evidence for the importance of NF-kB in the regulation of parturition. Emerging evidence also supports the importance of activator protein-1 (AP-1) in regulating the proteins involved in parturition. Activator protein-1 is required for transcriptional regulation of IL-8 in human myocytes. Several groups have reported on its activity in the myometrium at term and in labour. The physiological processes of term labour are similar to preterm labour and require three components; cervical ripening, membrane rupture and uterine contractility. Both NF-kB and AP-1 contribute to controlling the expression of the key proteins responsible for all three sequelae including, cytosolic phospholipase A2, cyclooxygenase-2 (COX-2) (which is the rate limiting enzyme for prostaglandin E2 synthesis), the oxytocin receptor and the interleukins IL-8 and IL-6. Premature activation of these transcription factors, by stimuli such as cytokines in the presence of inflammation, can therefore trigger preterm cervical dilatation and preterm rupture of membranes in the presence or absence of uterine contractility. A strong association exists between exposure to intrauterine inflammatory mediators and the risk of cerebral palsy, periventricular leucomalacia, and bronchopulmonary dysplasia. Given that NF-kB also regulates the expression of pro-inflammatory cytokines, premature activation of NF-kB can also worsen neonatal outcome. Both NF-kB and AP-1 are therefore attractive therapeutic targets for the prevention of preterm labour and for reducing adverse neonatal outcomes.

Sulfasalazine therefore appears to hold promise for potential use as a therapeutic agent for the prevention of inflammation-induced preterm labour. As it is readily available, affordable and has a good safety record in pregnancy we aimed to explore this further. We sought to determine the effect of supra-therapeutic (5 mM) and therapeutic (0.015 mM and 1 mM; equivalent to blood and stool concentrations following standard oral regimens) concentrations of SASP on basal and IL-1β-induced NF-kB and AP-1 activity in both amnion epithelial cells and myocytes in vitro. We then explored its effect on basal and IL-1β-induced production of key pro-inflammatory cytokines that are associated with intrauterine inflammation and the fetal inflammatory response syndrome.

Methods

Ethics statement

Placenta and myometrium were collected in accordance with Ethical approval Ref. 2002/6283 (Hammersmith, Queen Charlotte’s & Chelsea Hospitals Research Ethics Committee) and REF 3357 (Riverside Research Ethics Committee), respectively, and in accordance with Imperial College NHS Healthcare Trust Research and Development. Written consent was obtained from all subjects.

Reagents

Sulfasalazine was purchased from Sigma (St Louis, MO) and dissolved in RPMI-1640 (Invitrogen Life Technologies, Grand Island, NY) containing 1% fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Interleukin-1β was purchased from R&D Systems (Abingdon, UK). Antibodies and dilutions used for immunoblotting are shown in Table 1. Total RNA was extracted using Trizol (Invitrogen Life Technologies), and reagents for cDNA synthesis and quantitative RT-PCR were purchased from Sigma unless stated otherwise. Primers for gene amplification are shown in Table 2. The lactate dehydrogenase (LDH) assay was purchased from Cayman Chemical (Ann Arbor, MI).

Cell culture and treatment

Placenta and myometrial biopsies were collected at the time of pre-labour caesarean section. For amnion
epithelial cell culture, amnion was separated from the choriondecidua, cut into strips and washed in PBS. It was then incubated in 0.5 M of EDTA-PBS for 15 min at room temperature, and rinsed three times in PBS. The intracellular matrix was digested in 2 g/l of dispase for 45 min at 37°C. Epithelial cells were then isolated by shaking the amnion strips vigorously in Dulbecco’s modified Eagle’s medium (DMEM) for 4 min before being pelleted by centrifugation for 10 min at 900 g. Cells were resuspended in DMEM supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin and 100 µg/ml of streptomycin and grown to confluence in 2 ml of medium in six-well plates at 5% CO2.

For myocyte culture, myometrial biopsies were taken from the upper margin of lower segment incisions during elective caesarean sections. Tissue was rinsed in PBS and mechanically dissected using two sterile blades until a paste-like texture was formed. Cells were isolated by incubating 0.1 M collagenase 1A, 0.1 M collagenase XI and 5 mg/ml of BSA, dissolved in DMEM and DMEM–F12-Ham (DMEM-Ham), of equal portions for 45 min at 37°C. The suspension was filtered through a cell strainer and centrifuged at 400 g for 5 min and pelleted cells were resuspended and cultured in DMEM. Cells up to passage four were used. Upon final passage, cells were seeded into six-well culture plates and cultured in 2 ml of medium. Sulfasalazine was dissolved to the required concentration in RPMI medium, in line with previous studies. For all experiments non-SASP-treated cells were cultured in the same volume of RPMI medium to serve as a vehicle control. An initial dose response and time-course was performed with SASP treatment in amniocytes and myocytes. Pre-incubation with SASP was for 120 min for detection of NF-kB and COX-2, whereas pre-incubation for detection of phospho-c-Jun was for 30 min in amniocytes and 120 min in myocytes. The experiment was terminated by washing cells in ice-cold PBS followed by immediate storage at −80°C until further use.

Protein extraction and SDS–PAGE and Western blotting

Protein was extracted with whole cell lysis buffer (Cell Signaling, Beverly, MA) containing 5 µl/ml of protease inhibitor (Sigma). Cells were incubated with ice-cold buffer for 5 min and centrifuged for 20 min at 16 000 g. Before SDS–PAGE, protein concentrations were determined using the Bio-Rad quantification assay measuring absorbance at 655 nm (Bio-Rad, Hercules, CA). Protein was resolved by SDS–PAGE and subsequently transferred onto PVDF membranes (GE Healthcare, Chalfont St Giles, UK) at 100 V (constant voltage). Membranes were then blocked in 5% (weight/volume) milk in Tris-buffered saline supplemented with 0.1% Tween 20 for 1 hr before being probed with the relevant primary and secondary antibodies under the conditions specified in Table 1. Chemiluminescence detection was performed with ECL Plus (GE Healthcare) and imaged using the chemiluminescent imager (GE Healthcare). Blots were scanned and densitometry performed with IMAGEJ (v1.44p), U.S. National Institutes of Health, Bethesda, MD.
Cytokine mRNA quantification by quantitative RT-PCR

Total RNA was isolated from cultured cells with Trizol® according to the manufacturer’s instructions. Two microgrammes of RNA was incubated with 1 μl of DNase and 1 μl of 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 of 10 mM dNTP and 1 μl of Oligo-dT for 10 min at 70°C. To this mix, 2 μl of × 10 M-MLV RT buffer, 1 μl of M-MLV reverse transcriptase, 0.5 μl of RNase inhibitor and 6.5 μl of dH2O were added and incubated at room temperature for 15 min, 37°C for 50 min before the reaction was terminated by incubating at 80°C for 10 min. Samples were stored at −20°C until further use. Relative quantification of gene expression was performed using real-time PCR performed on an Applied Biosystems StepOne™ Real-Time PCR System using SYBR® Green Master mix (Applied Biosystems, Foster City, CA). Water non-template controls were used. Primer efficiency was first established; with efficiencies of between 94 and 100% for primers sets used. Gel electrophoresis was also used to confirm the correct size of the single amplified product. Relative quantification was performed using the comparative C_T method (ΔΔ C_T). Fold change from control samples at the −2-hr time-point were taken relative to the housekeeping gene, L19, GAPDH and β-actin housekeeping genes were used and target genes were analysed relative to all three housekeeping genes at each time-point. Data presented are relative to the L19 gene in amniocytes and β-actin in myocytes. Table 2 provides the primer sequences for the relevant target genes.

Determination of cell integrity and viability

The potential cytotoxic effects of SASP were assessed by examining cell morphology of treated cells using standard light microscopy. Cell viability was determined using a trypan blue dye exclusion test. Stained cells were quantified using the Countess® Automated cell counter (Invitrogen Life Technologies). Cell membrane integrity was determined by measuring LDH release into culture medium using the LDH assay kit (Cayman Chemicals) as per the manufacturer’s instructions. Triton-X (1%) -treated cells were used as a positive control.

Statistical analysis

Experimental groups consisted of three to six biological replicates for protein detection studies and three biological replicates for interleukin and LDH detection. Numbers are specified for each experiment in the figure legend of each data set. Statistical analysis was performed with GraphPad Prism v5 (GraphPad Software, San Diego, CA). A one-way analysis of variance (ANOVA), ANOVA of repeated measures or a two-way ANOVA of repeated measures was used depending on the number of treatment groups/tests applied to one sample. The Bonferroni’s multiple comparisons test was applied.

Results

A supra-therapeutic concentration of SASP is required to inhibit IL-1β-induced NF-κB activation but has no effect on COX-2 protein expression in cultured amniocytes

A dose–response and time–course with SASP was first performed in cultured amniocytes. Cells were pre-incubated with SASP at 0.1 mM, 1 mM or 5 mM for 30, 60 and 120 min before the addition of 1 ng/ml of IL-1β for 15 min. Although SASP treatment did not alter basal NF-κB phosphorylation, 5 mM SASP was sufficient to inhibit IL-1β-induced phosphorylation of NF-κB at all time-points (Fig. 1a–c). A therapeutic concentration of 0.015 mM failed to inhibit IL-1β-induced phospho-p65 (Fig. 1e).

As NF-κB regulates the expression of COX-2, the effect of SASP on basal and IL-1β-induced COX-2 protein levels was examined in treated amniocytes. Cells were pre-incubated with SASP for 120 min to achieve maximal NF-κB inhibition, followed by IL-1β for 4 hr. Treatment with SASP had no effect on basal or IL-1β-induced COX-2 protein expression levels in treated cells (Fig. 1d,f).

A supra-therapeutic concentration of SASP is required to inhibit IL-1β-induced pro-inflammatory cytokines in amniocytes

To determine the effect of SASP on IL-1β-induced cytokine production in cultured amniocytes, cells were pre-incubated with 5 mM of SASP or medium vehicle control (time-point 2) for 2 hr before the addition of IL-1β or PBS control at time-point 0. RNA was extracted at the following time-points; −2 (before SASP treatment), 0 (time of IL-1β addition), and 4, 8, 12 and 24 hr after IL-1β addition and changes in IL-6, IL-8 and TNF-α were determined by quantitative RT-PCR (Fig. 2a–c). Target gene expression was the same regardless of which housekeeping gene was used. Interleukin-1β significantly induced the expression of IL-6 at 6 hr, peaking by 100-fold at the 8-hr time-point (P < 0.05), the effect of which was inhibited by pre-incubation with 5 mM of SASP (Fig. 2a). Interleukin-8 mRNA expression increased 450-fold 4 hr after IL-1β addition (P < 0.0001), and pre-incubation with 5 mM SASP significantly suppressed this
effect ($P < 0.0001$) (Fig. 2b). Similarly, treatment of amniocytes with IL-1β significantly up-regulated the expression of TNF-α at 4 hr by 16-fold ($P < 0.001$), the effect of which was again attenuated by pre-incubation with 5 mM SASP ($P < 0.001$) (Fig. 2c).

At the therapeutic concentrations of 0·015 mM and 1 mM, no attenuation of IL-1β-induced IL-6, IL-8 and TNF-α was seen. Instead IL-1β treatment with 0·015 mM of SASP synergistically increased IL-6 ($P < 0.01$), IL-8 ($P < 0.05$) and TNF-α ($P < 0.05$) (Fig. 3a–c).

**Sulfasalazine causes a rapid transient basal increase in pro-inflammatory cytokines and AP-1 activation in amniocytes**

Contrary to what was expected, pre-incubation with 5 mM of SASP alone significantly increased basal mRNA expression of all pro-inflammatory cytokines (Fig 2d–f) with levels of IL-6 mRNA increased by 80-fold (Fig. 2d) ($P < 0.01$) compared with vehicle control. Pre-incubation of cells similarly caused a transient increase in IL-8 and
TNF-α mRNA (Fig. 2e,f). An increase in basal IL-6, IL-8 and TNF-α was also seen with 1 mM of SASP, but was not statistically significant (Fig. 3d–f).

Both COX-2 and the pro-inflammatory cytokines IL-6, IL-8 and TNF-α can be transcriptionally regulated by the pro-inflammatory transcription factor AP-1. To test the effect of SASP on AP-1 activation, cells were pre-incubated with 0, 1 mM, 5 mM or 10 mM SASP for 30, 60 and 120 min and stimulated with 1 ng/ml of IL-1β for 15 min (Fig. 4). Activation of AP-1 was determined by immunoblotting for phospho-c-Jun from whole cell protein extract. A significant increase in basal and IL-1β-induced AP-1 activity was seen 30 min post-incubation with SASP 5 mM (P < 0.01 and P < 0.05, respectively). This response was transient with levels of phosphorylated c-Jun in SASP-treated cells comparable to non-stimulated controls by 60 and 120 min post-treatment.

A supra-therapeutic concentration of SASP is required to inhibit IL-1β-induced NF-κB and reduce COX-2 protein expression in cultured myocytes

A dose–response and time-course with SASP was performed in cultured myocytes to examine the effects on...
NF-κB activation. As for amniocytes, cells were pre-incubated with SASP 0·015 mM, 1 mM or 5 mM for 30, 60 and 120 min before the addition of 1 ng/ml of IL-1β for 15 min; 5 mM of SASP was required to inhibit IL-1β-induced phosphorylation of NF-κB at all time-points (Fig. 3a–c,e).

Myocytes pre-incubated with SASP 5 mM for 2 hr, before a 4-hr stimulation with IL-1β showed significantly less expression of COX-2 compared with control-treated myocytes \((P < 0·05)\) (Fig. 3d). Lower concentrations of SASP (0·015 mM and 1 mM) failed to inhibit COX-2 expression (Fig. 3f).

Sulfasalazine induces pro-inflammatory cytokine production and has a differential effect on AP-1 dependent on concentration in myocytes

The effect of 0·015 mM and 1 mM SASP on pro-inflammatory cytokines was determined by RT-PCR using β-actin as the housekeeping gene. Neither concentration was able to inhibit IL-1β-induced cytokines (Fig. 4a–c), yet an augmented IL-8 response was seen with the combination of SASP 0·015 mM and IL-1β \((P < 0·05)\) (Fig. 4c). Although SASP 1 mM led to an increase in basal IL-6, IL-8 and TNF-α production, this was not statistically significant (Fig. 4d–f).
Activation of AP-1 was examined in myocytes pre-incubated with SASP for 30, 60 or 120 min before treatment with 1 ng/ml of IL-1β (Fig. 7a–c). Sulfasalazine did not cause an increase in basal AP-1 activity, and contrary to the effect seen in amniocytes, SASP 5 mM inhibited IL-1β-induced AP-1 phosphorylation (P < 0.01). At the lower concentration of 0.015 mM, an augmented response was seen in combination with IL-1β stimulation, leading to increased AP-1 phosphorylation (Fig. 7d).

Sulfasalazine has significant cytotoxic effects

Cell viability of amniocytes and myocytes following treatment with supra-therapeutic SASP (5 mM) was determined by the trypan blue dye exclusion test. The total number of cells present in the vehicle control groups did not differ significantly from that in SASP-treated groups. Figs 8(a) and 9(a) show the morphological appearance of representative amniocytes and myocytes, respectively, along with percentage viability at time-points 0, 2, 6, 10, 14 and 26 hr following treatment with SASP 5 mM. These time-points correspond to the −2, 0, 4, 8, 12 and 24 hr time-points in the cytokine detection studies. Although cell viability and morphology of amniocytes were not significantly altered with SASP treatment, the viability of myocytes was reduced from as early as 2 hr after incubation with SASP 5 mM. Alterations in myocyte morphology could be detected in cells treated with 5 mM SASP following 6 hr of incubation.

A dose–response was performed with 0.015 mM, 1 mM and 5 mM (with non-stimulated vehicle control) to assess cytotoxic effects. Cell morphology was examined along with LDH production in the medium to assess the cell membrane integrity of amniocytes and myocytes. The LDH was significantly increased over time with 5 mM SASP in both amniocytes (P < 0.001) and myocytes (P < 0.001), (Figs 8b and 9b, respectively, with higher concentrations seen in myocytes). No significant increase was seen with the therapeutic concentrations or vehicle control.

**Discussion**

A firm causal link exists between the presence of inflammation and both preterm labour and poor neonatal outcome. Accordingly, research efforts have been made to
A synergistic increase in COX-2 expression was before the addition of IL-1 expression, cells were pre-treated for 120 min are shown for 30 min of pre-incubation (a), 0 min (b) and 120 min (c). For COX-2 expression, cells were pre-treated for 120 min before the addition of IL-1β for 15 min. Representative immunoblots are shown for 30 min of pre-incubation (a), 60 min (b) and 120 min (c). For COX-2 expression, cells were pre-treated for 120 min before the addition of IL-1β for 4 hr (d). Subsequent experiments were performed to determine the effects of therapeutic concentrations 0-0.015 mM and 1 mM on p-p65 (e) and COX-2 (f). Immunoblots were probed for β-actin as an internal loading control. Densitometric analysis of the immunoblots was conducted revealing a significant inhibition of IL-1β-induced p-p65 (P < 0.01) at 5 mM (c). No significant inhibition was seen at the lower concentrations of 0.015 mM or 1 mM (e). Densitometric analysis of the immunoblots was conducted revealing a significant inhibition of IL-1β-induced COX-2 expression (P < 0.05) with high concentration SASP (d), but not the lower concentrations of 0.015 mM or 1 mM (f). A synergistic increase in COX-2 expression was seen upon treatment with both IL-1β and SASP 0.015 mM (f). For statistical analysis, analysis of variance (ANOVA) of repeated measures (p-p65) and one-way ANOVA was used (COX-2) with Bonferroni’s multiple comparison test, *P < 0.05, **P < 0.01. n = 3.

Figure 5. A supra-therapeutic concentration of sulfasalazine (SASP) is required to inhibit interleukin-1β (IL-1β) -induced nuclear factor-κB (NF-κB) activation and cyclo-oxygenase-2 (COX-2) protein levels in cultured myocytes. Protein was extracted from IL-1β-stimulated and SASP-treated cells and whole cell phosphorylated p65 (p-p65) was examined using immunoblotting. Myocytes were pre-incubated with 0-1, 1 or 5 mM of SASP for either 30, 60 or 120 min before the addition of 1 ng/ml of IL-1β for 15 min. Representative immunoblots are shown for 30 min of pre-incubation (a), 60 min (b) and 120 min (c). For COX-2 expression, cells were pre-treated for 120 min before the addition of IL-1β for 4 hr (d). Subsequent experiments were performed to determine the effects of therapeutic concentrations 0-0.015 mM and 1 mM on p-p65 (e) and COX-2 (f). Immunoblots were probed for β-actin as an internal loading control. Densitometric analysis of the immunoblots was conducted revealing a significant inhibition of IL-1β-induced p-p65 (P < 0.01) at 5 mM (c). No significant inhibition was seen at the lower concentrations of 0.015 mM or 1 mM (e). Densitometric analysis of the immunoblots was conducted revealing a significant inhibition of IL-1β-induced COX-2 expression (P < 0.05) with high concentration SASP (d), but not the lower concentrations of 0.015 mM or 1 mM (f). A synergistic increase in COX-2 expression was seen upon treatment with both IL-1β and SASP 0.015 mM (f). For statistical analysis, analysis of variance (ANOVA) of repeated measures (p-p65) and one-way ANOVA was used (COX-2) with Bonferroni’s multiple comparison test, *P < 0.05, **P < 0.01. n = 3.

explore the potential use of anti-inflammatory drugs to prevent preterm birth including progesterone, COX-2 inhibitors, N-acetyl cysteine and the anti-inflammatory prostaglandin 15-deoxy-A2,14 prostaglandin J2,44,51-57 Sulfasalazine holds potential advantages over these drugs because it is generally well tolerated, and already has an established safety profile in pregnancy.16 Additionally it has been shown to inhibit NF-κB,58 the transcriptional regulator of both contractile-associated proteins and inflammatory cytokines.59 As levels of IL-1β are increased in fetal membranes during preterm and term labour, and even more so in the presence of chorioamnionitis,59 IL-1β is commonly used as an inflammatory stimulus in experimental models of infection/inflammation-induced preterm labour.

Sulfasalazine, rather than its metabolites sulfapyridine or 5-ASA, is the active component required for the inhibition of NF-κB. At similar concentrations to sulfasalazine, neither the anti-inflammatory metabolite 5-ASA nor the antibiotic metabolite sulfapyridine is able to inhibit NF-κB activity in SW620 colonic epithelial cells.49 As little as 30% of SASP is absorbed in its unaltered...
form, with the rest being degraded by colonic bacteria and azo-reduced to its metabolites. Although the concentrations within the fetal membranes and myometrium of pregnant women taking SASP are unknown, it is found as an intact molecule in synovial fluid of inflamed joints at comparable levels to blood plasma of 5–8 μg/ml when used in the treatment of rheumatoid arthritis. This equates to between 0.01 and 0.02 mM, far less than the concentration required to inhibit NF-κB activity in vitro in the majority of studies.

Sulfasalazine has been shown to inhibit NF-κB-induced activation via multiple stimuli including PMA, TNF-α and LPS. Treatment of amniocytes and myocytes with IL-1β leads to a rapid phosphorylation/activation of inhibitor of NF-κB kinase α (IKKα) and IKKβ and subsequently the phosphorylation and degradation of IkBα, phosphorylation of the p65 subunit and translocation into the nucleus. Phosphorylation of p65 at Ser 536 is mediated by IKKβ and/or IKKα and is required for efficient NF-κB-mediated transcription. For this reason we used phospho-p65 (Ser 536) as a marker of NF-κB activation for examining the effect of SASP on basal and IL-1β-stimulated amniocytes and myocytes (Figs 1 and 5, respectively).
A supra-therapeutic concentration of sulfasalazine (SASP) is required to inhibit activator protein-1 (AP-1) activation in cultured myocytes. Protein was extracted from interleukin-1β (IL-1β)-stimulated and SASP-treated cells and whole cell phosphorylated c-Jun (p-c-Jun) was examined using immunoblotting. Myocytes were pre-incubated with 0-1, 1 or 5 mM of SASP for either 30, 60 or 120 min before the addition of 1 ng/ml of IL-1β for 15 min. Representative immunoblots are shown for 30 min of pre-incubation (a), 60 min (b), and 120 min (c). A subsequent experiment was performed to determine the effect of therapeutic concentrations of 0·015 mM and IL-1β-stimulated p-c-Jun (d) Immunoblots were probed for β-actin as an internal loading control. Den- sitometric analysis of the immunoblots was conducted revealing a significant inhibition of IL-1β-induced p-c-Jun after 60 and 120 min of pre-incubation (P < 0·01) (b, c). A synergistic increase in AP-1 activation was seen with a 120-min pre-incubation of cells with SASP 0·015 mM and IL-1β stimulation (P < 0·01) (d) For statistical analysis, analysis of variance of repeated measures with Bonferroni’s multiple comparison test was used, *P < 0·05, **P < 0·01, ***P < 0·001, n = 3–4.

We performed dose–response and time–course analyses to determine the lowest concentration required to inhibit nuclear factor–κB (NF-κB) at the cellular level. A supra-therapeutic concentration of 5 mM was required in both amniocytes and myocytes to achieve inhibition of IL-1β-stimulated NF-κB. This was consistent with SASP concentrations previously reported to inhibit LPS-induced NF-κB activation in fetal membrane tissue explants.20,21,50,65,66 We had anticipated a lower concentration to be effective in cells because treatment of colonic epithelial cells with 0·5 mM shows a 50% reduction in NF-κB activation,49 and a reduction is seen with 1 mM in SASP-treated Jurkat cells.57

As NF-κB regulates and is regulated by IL-6, IL-8 and TNF-α, the effect of SASP on their expression in IL-1β-stimulated amniocytes was therefore determined. Only the supra-therapeutic 5 mM concentration was able to attenuate IL-1β-induced mRNA expression of IL-6, IL-8 and TNF-α in amniocytes in a mechanism likely to be dependent on NF-κB inhibition; because the lower therapeutic concentrations neither inhibited NF-κB nor IL-1β-induced cytokines in amniocytes and myocytes (Figs 3a–c and 6a–c, respectively). This is consistent with the findings of Lappas et al.,20 who demonstrated that 5 mM of SASP was the minimum concentration required not only to inhibit LPS-induced NF-κB activation but also to inhibit LPS-induced IL-6, IL-8 and TNF-α production in fetal membrane explants.

Surprisingly, both supra-therapeutic (Fig. 2d–f) and therapeutic (Figs 3d–f and 6d–f) concentrations of SASP possessed pro-inflammatory properties in amniocytes and myocytes. Previous studies examining the effect of SASP on LPS-induced interleukin production by fetal membranes have demonstrated only anti-inflammatory effects.20,21 However, both studies used LPS as a stimulant with no pre-incubation step, and did not report on the effect of SASP alone on interleukin production. This unexpected increase in pro-inflammatory cytokine production led us to explore the effect of SASP on AP-1, another transcription factor known to regulate the expression of inflammatory response genes. We demonstrated a rapid increase in phospho-c-Jun 30 min after treatment with 5 mM of SASP, the effect of which disappeared by 120 min (Fig. 4a–c). This could explain the rapid transient increase in basal cytokines in amniocytes (Fig. 3d–f). A moderate increase in non-stimulated and
IL-1β-induced phospho-c-Jun was observed in amniocytes following 1 mM SASP pre-incubation; which is likely to be responsible for increased basal and IL-1β-induced IL-6, IL-8 and TNF-α (Fig. 3a–c). Similarly, in myocytes pre-incubated with 0.015 mM SASP before IL-1β stimulation, an augmented response was seen in both phospho-c-Jun and IL-8 production (Figs 7d and 6b). This is consistent with the moderate activation of AP-1 DNA-binding activity previously demonstrated in SW620 human colonic epithelial cells pre-incubated with SASP before the addition of TNF-α.49 This temporal induction of cytokines seen in our study may be explained by this rapid and transient activation of AP-1, which is known to play a role in the transcriptional regulation of the IL-6, IL-8 and TNF-α genes.32,68,69

There is mounting evidence that AP-1 may also play an important role in the activation of the parturition cascade. We have previously shown that AP-1 activation in the murine uterus by a specific LPS serotype alone is sufficient to drive an inflammatory cascade that leads to preterm labour.35 Even in the absence of increased NF-κB activity, the rapid induction of AP-1 activation was associated with increased mRNA expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α, as well as contractile associated proteins including Con 43,70,71 oxytocin receptor40 and COX-2.38,72 The AP-1 subunits have previously been shown to be up-regulated in the myometrium of women in preterm labour.34 Therefore, an unintended activation of AP-1 by SASP could worsen outcome in the context of pregnancy and prevention of preterm labour.

In the current study, despite the inhibition of both basal and IL-1β-induced NF-κB with 5 mM of SASP, no inhibition was seen in COX-2 protein expression in amniocytes (Fig. 1). This result is consistent with findings from Lappas et al.,50 where SASP concentrations of up to 10 mM failed to inhibit COX-2 in LPS-treated amnion, choriodedua or placental explants. The promoter region of the COX-2 gene contains binding sites for NF-κB, AP-1 and cAMP-response elements.73 These transcription factors bind in a variety of combinations depending on cell type and also on the regulatory pathway that has been activated.74 Both NF-κB and AP-1 have been shown to be required for IL-1β-induced up-regulation of COX-2 in amnion epithelial cells.38 Therefore, the opposing effects of SASP-induced inhibition of NF-κB and activation of AP-1 make the overall effect on COX-2 transcription difficult to resolve. The effect of SASP on alternative contraction-associated proteins (cytosolic phospholipase A2α and Con-43 in amniocytes, and oxytocin receptor and Con-43 in myocytes) were not examined because no increase in mRNA was demonstrated with IL-1β (see Supplementary material, Fig. S1).

Figure 8. Assessment of sulfasalazine (SASP) toxicity in amniocytes. Amniocytes were incubated with 0.015, 1 and 5 mM of SASP for 120 min at time -2 to mimic experimental time-points previously used. At each time-point cells were imaged by microscopy at all concentrations, and percentage cell viability was assessed with Trypan blue for cells treated with 5 mM of SASP (a). No significant effect was seen in cell viability with treatment with SASP. In a separate experiment, medium was assayed at each time-point for lactate dehydrogenase (LDH) (b). LDH release was significantly higher from the 8 hr time-point onwards in amniocytes treated with the supra-therapeutic concentration of 5 mM (P < 0.01 at 8 and 24 hr, and P < 0.001 at 12 hr). No significant increase in LDH production was seen over time with the lower concentrations of 0.015 mM or 1 mM. For statistical analysis a two-way analysis of variance of repeated measures with Bonferroni’s multiple comparison test was used, ***P < 0.001, **P < 0.01. n = 3.

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Our study also revealed that supra-therapeutic concentrations of SASP had significant cytotoxic effects on myocytes and amniocytes, whereas therapeutic doses did not (Figs 8 and 9). The SASP-induced cell death of amniocytes and myocytes would probably be detrimental for the treatment of pregnant women because apoptosis is associated with preterm rupture of membranes.75

The observation that the treatment of Crohn’s disease in pregnancy with 5-ASA/SASP leads to a 50% reduction in preterm delivery rates compared with untreated controls17 requires further investigation. It is unlikely that this protective effect is the result of direct action of SASP upon gestational tissues. Although we do not suggest stopping SASP treatment for Crohn’s disease in pregnancy, our data do not support the use of SASP for the prevention of inflammation-induced preterm labour. This is because the therapeutic concentration of SASP fails to inhibit the induction of key labour-associated transcription factors NF-κB and AP-1 by IL-1β, and leads to pro-inflammatory cytokine production. Additionally, although supra-therapeutic concentrations are capable of inhibiting IL-1β-induced NF-κB and AP-1, this is also associated with significant cytotoxic effects.

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Author contributions
LS, PRB designed the study, LS, KT, EB, ZBMR and YL performed the experiments and LS, KT, DAM, TGT and PRB wrote the paper.

Disclosures
The authors have no financial disclosures or competing interests.

References


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**Supporting Information**

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

**Figure S1.** The effect of interleukin-1β (IL-1β) on contraction-associated gene expression was examined by quantitative RT-PCR using L-19 and β-actin as the housekeeping genes for amniocytes and myocytes, respectively.