Reproductive Biology and Endocrinology

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Lipopolysaccharide induces nitric oxide synthase expression and platelet-activating factor increases nitric oxide production in human fetal membranes in culture

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Published: 10 June 2004

Reproductive Biology and Endocrinology 2004, **2**:29

Received: 17 March 2004
Accepted: 10 June 2004

This article is available from: http://www.rbej.com/content/2/1/29

Abstract

Background: Platelet-activating factor and nitric oxide may be involved in the initiation of human labour as inflammatory mediators. The aim of this study was to test whether platelet-activating factor and lipopolysaccharide were able to induce nitric oxide synthase expression and stimulate the production of nitric oxide in human fetal membrane explants in culture.

Methods: Fetal membranes were collected from Caesarean sections at term. RNA was extracted from membranes and subjected to a qualitative RT-PCR to assess the baseline expression of iNOS. Discs of fetal membranes were cultured for 24 hours in the presence of platelet-activating factor at a dose range of 0.1 nanomolar – 1 micomolar or 1 microgram/ml lipopolysaccharide. Nitric oxide production was measured via nitrite ions in the culture medium and mRNA for iNOS was detected by RT-PCR.

Results: Culturing the membrane discs in medium containing serum induced nitric oxide synthase expression and platelet-activating factor significantly stimulated the production of nitric oxide under these conditions. When cultured without serum inducible nitric oxide synthase expression was induced by lipopolysaccharide, but not by platelet-activating factor.

Conclusion: Platelet-activating factor may have a role in the initiation of labour, at term or preterm, via the increased local production of nitric oxide as an inflammatory mediator. In this model of intrauterine infection, lipopolysaccharide was found to induce iNOS expression by fetal membranes, and this mechanism could be involved in preterm labour.

Background

Preterm birth is a major obstetric problem, affecting up to 10% of births in the UK and USA [1]. Intrauterine infection is a known risk factor for preterm labour and is associated with the pathology of the condition [2-4]. Bacteria and bacterial products, such as lipopolysaccharide (LPS) induce the production of inflammatory mediators, such as prostaglandin E_2 and $F_{2\alpha}$ [5] and IL-6 and IL-8 [6], by

intact fetal membranes in culture. Platelet-activating factor (PAF) may also have a role in preterm labour and increased levels of PAF in amniotic fluid (up to 44.1 ng/ml, 79.9 nM) before 37 weeks of gestation have been associated with premature labour [7]. The exact mechanisms involved in the initiation of human parturition have yet to be fully elucidated. There is growing evidence for an inflammatory mechanism in the onset of labour at term [8] and preterm [9,10], where inflammatory mediators such as PAF and nitric oxide (NO) may be involved. It has been thought for some time that PAF could be involved with the onset of parturition [11,12], as PAF is associated with fetal lung maturation and is produced by type II pneumocytes, along with surfactant [12,13]. It is transferred from the fluid in the lungs into the amniotic fluid in late gestation, by diffusion or fetal breathing movements [11,12]. Levels of PAF are thought to increase in the amniotic fluid towards term [14-16]. The mean concentration of PAF in samples of amniotic fluid obtained after spontaneous labour and vaginal delivery at term has been determined as 0.1 nM [17], with a form identified with an octadecyl side chain [15]. PAF is a highly potent inflammatory mediator [18] and has been shown to stimulate the production of prostaglandins from fetal membranes [19]. A link between PAF and iNOS in other tissues has been suggested by Qu et al. [20] and PAF has been found to evoke release of NO from human endometrium [21,22]. The local application of PAF was found to induce cervical ripening in rats, via infiltration of polymorphonuclear leukocytes [23]. Nitric oxide has also been found to be a mediator of cervical ripening, via an inflammatory mechanism [24], suggesting a role in promoting parturition.

It is well established that inducible nitric oxide synthase (iNOS) can liberate high concentrations of NO locally [25]. This enzyme is not expressed constitutively and is normally absent from cells until they are stimulated by cytokines or growth factors [26,27]. Butyrate, a metabolite produced by some anaerobic bacteria, has been shown to promote the expression of iNOS in a cell line in culture [28]. Expression of iNOS mRNA has been previously identified in human fetal membranes at term, specifically in the amnion, chorio-decidua and placenta [29]. Immunostaining has shown the presence of iNOS in fibroblasts of the mesenchyme of amnion and chorion and in decidual macrophages [30]. NO metabolite concentrations were found to be higher in amniotic fluid collected from labouring compared to non-labouring women, both at term and preterm [31]. Increased staining for iNOS was detected in trophoblast cells from women in labour compared to non-labouring [31], however Kakui et al. [32] reported no difference in iNOS expression in placental tissues for women in labour compared to those not in labour. Upregulated expression of iNOS was reported in human cervical biopsies obtained during the third trimester compared to the first trimester [33].

A robust model for maintaining human fetal membrane explant discs (comprising amnion, chorion and decidua) in culture has been established and validated by Sullivan [19]. It is common practice in tissue culture to include serum in the culture medium, however it has been found that exposure to serum can induce the expression of some enzymes, such as COX-2 [34]. For this reason, intact fetal membrane explants were used in this study with and without serum in the culture medium.

The aim of this study was to investigate whether PAF and LPS were able to induce iNOS mRNA expression and to stimulate the production of nitric oxide in intact human fetal membrane explants in culture.

Methods

Tissue collection

Human fetal membranes were obtained from elective Caesarian sections at term (n = 22; pre-labour), with informed consent and the ethical approval of the Wolverhampton District Local Research Ethics Committee, in compliance with the Helsinki Declaration. Four samples of fetal membranes were collected after normal vaginal delivery, to provide a post-labour comparison. The pregnancies and deliveries were free of any complications and were singletons. It was not known whether antibiotics were administered to the women before delivery.

Tissue culture

Following collection, the pre-labour membranes were washed with sterile phosphate buffered saline (PBS, pH 7.4) and discs 2.5 cm in diameter were punched out. Some sample discs of the fresh membranes were immediately homogenised and the RNA extracted by the guanidium thiocyanate-phenol method (TRI Reagent®) for subsequent analysis. 12 disks were washed twice with sterile PBS and transferred to two 6 well plates containing 2 ml HAM'S F-12 medium, with 50 units of penicillin and 50 μg streptomycin per ml. Six sets of the membrane samples were cultured with 10% fetal calf serum and nine sets were cultured without serum in the culture medium (Figure 1). The discs were cultured for 24 hours at 37°C in 5% CO_2 in humid air, to allow for recovery. Following this, the medium was removed and replaced with either fresh medium (control, n = 4) or medium containing 1 μg/ml LPS (n = 4) or medium containing 1 μg/ml LPS + 2 mM sodium butyrate (n = 4) (Figure 1). For a further 5 samples of membranes, the medium was removed and replaced with either fresh medium (control, n = 5) or medium containing PAF at 0.1 nM, 1.0 nM, 10 nM, 100 nM or 1.0 μM (n = 5) (Figure 1). All concentrations were chosen to be consistent with other published studies and to cover the

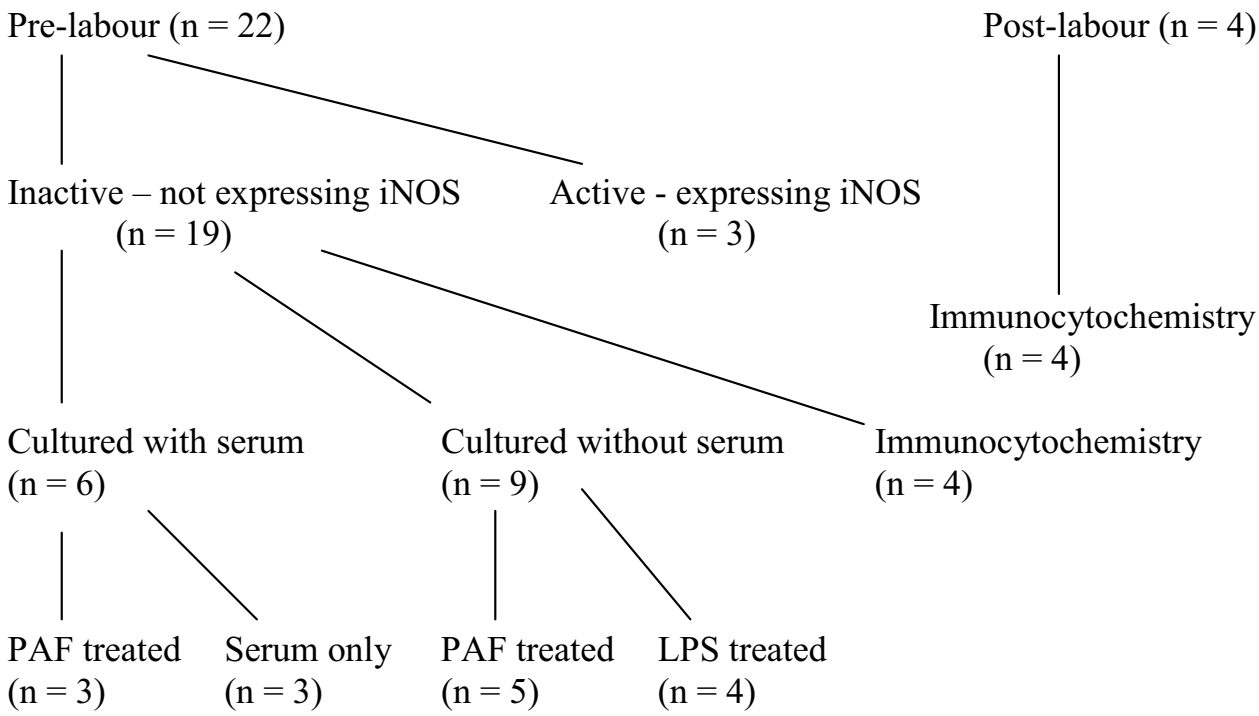

Figure 1
Summary of the fetal membrane samples and protocols used.

physiological range. For the membrane discs cultured with serum, control (n = 3) and PAF at 1.0 μM (n = 3) only were used (Figure 1). As PAF is extremely lipid soluble, solutions were made up using culture medium, which contained sufficient surfactants to act as carriers for PAF. After 24 hours in culture the membrane discs and supernatants were removed from each well. The time period for culture was selected following a time profile study. The discs were immediately homogenised and the RNA extracted using TRI Reagent® for subsequent analysis. The concentration of nitrite ions (NO_2^-), an oxidised product of NO, was measured immediately in the supernatants by a standard Griess reagent assay [35]. Culture medium was used as a blank in the assay to allow for the content of NO_2^-. The viability of the cells comprising the membrane discs in culture was assessed by a standard diaphorase staining technique [5,19].

RT-PCR for iNOS

The presence of specific mRNA for iNOS in the membrane discs was detected by a qualitative reverse transcription-polymerase chain reaction (RT-PCR) method. For the reverse transcription 2 μl of total fetal membrane RNA (approx. 400 μg/ml) was reverse-transcribed to cDNA using an AMV-Reverse Transcription System (Promega, Southampton, UK). The reverse transcription was performed according to the manufacturer's protocol using random hexamer primers. The PCR primers used were: 5'-GAGCTTCTACCTCAAGCTATC-3' (iNOS sense, bases 517–537) and 5'-TGATGTTGCCATTGTTGGTGG-3' (iNOS antisense, bases 806–826 of the human iNOS cDNA). The PCR conditions were: 2 m 30 s at 94°C, 45 s at 60°C, 5 m 30 s at 72°C (45 cycles). The reaction mix was: 10x PCR buffer (Stratagene, Cambridge, UK) 2.00 μl, dNTP Mix (Promega, Southampton, UK) 10 mM 0.50 μl, Sense primer (Oswel, Southampton, UK) 10 μM 0.40 μl, Antisense primer (Oswel, Southampton, UK), 10 μM 0.40 μl, Taq 2000™ polymerase (Stratagene, Cambridge, UK) 5 units/μl 0.04 μl, Nuclease-free water (Promega, Southampton, UK) 16.50 μl, cDNA template 2.00 μl. Total volume 21.84 μl.

Nested RT-PCR for iNOS

The amplified product was confirmed as human iNOS DNA by specific nested RT-PCR and DNA sequencing. Nested RT-PCR was used to detect low-level expression of

Table 1: Summary of results for iNOS expression and NO production for fetal membrane disks cultured in the absence or presence of serum

	Serum Free		With Serum	
	LPS	PAF	LPS	PAF
iNOS expression	Induction (n = 4)	No induction (n = 5)	N/A*	N/A*
NO production	No increase (n = 4)	No increase (n = 5)	N/A*	Increase (n = 3)

* These experiments were not performed as culturing with serum was found to induce iNOS and LPS was able to induce iNOS in the absence of serum

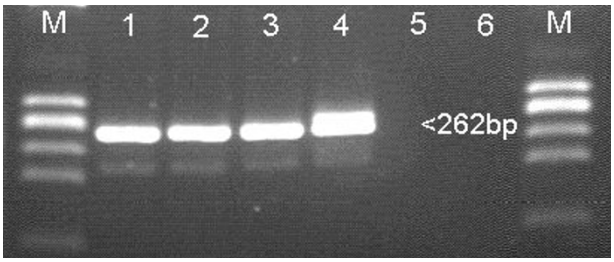

Figure 2
Detection of low iNOS mRNA levels in fetal membranes (FM) by nested RT-PCR, in serum-free cultures. Lane 1 fetal membranes immediately after collection, lane 2 control (cultured membranes without PAF), lane 3 10 nM PAF, lane 4 positive control (CHOiNOS cells), lane 5 PCR blank (no template), lane 6 empty, lanes M ΦX174 DNA/Hae III marker. The expected size of the product was 262 base pairs. The faint band below the main band at 262 base pairs was present in all gels and appeared to be an artefact of nested amplification. This is one example of four identical results with four different samples of fetal membranes.

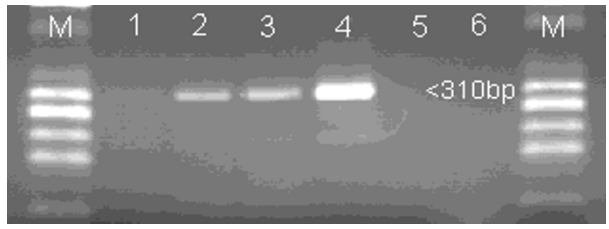

Figure 3
Induction of iNOS mRNA expression in human fetal membranes (FM) by culturing in the presence of serum for 24 hours. Lane 1 fetal membranes immediately after collection, lane 2 membranes cultured with serum, lane 3 membranes cultured with serum and 1 μM PAF, lane 4 positive control for iNOS, lane 5 PCR blank, M DNA markers. The expected size of the product was 310 base pairs. One example is shown of three identical results with three different samples of fetal membranes. All of the fetal membrane samples were initially negative for iNOS mRNA, as detected by the standard RT-PCR method.

iNOS mRNA. The designed nested primers used were: sense primer (5'-GTCAACCAATATTACGGCTC-3'), positioned between bases 544–563; antisense primer (5'-AGTAACGCACGTGTCTGCAG-3'), located between bases 786–805 of the human iNOS cDNA. The PCR conditions were the same as for the standard RT-PCR method. PCR products were fractionated by gel electrophoresis using a 2% agarose gel stained with ethidium bromide. Molecular weight markers for DNA, ΦX174 DNA/Hae III Marker (Promega, Southampton, UK), were run on the gels for reference. A cell line of Chinese hamster ovary cells transfected with the gene for human iNOS, so that they constitutively expressed iNOS (CHOiNOS), were used as a positive control for the RT-PCR method [28].

Immunohistochemistry

Human fetal membranes were collected after normal vaginal delivery (post-labour, n = 4) or at Caesarean section at term (pre-labour, n = 4) (Figure 1). Squares of 1 cm^2 were cut from human fetal membranes, at sites close to the placenta. The sample sections were snap-frozen immediately in a slush of dry ice in isopentane. Microsections of 6 μm thickness were cut at -20°C using a cryostat microtome. The sections were mounted on Vectabond-coated slides and immediately fixed in acetone. For immunohistochemical staining, the primary antibody was a monoclonal anti-human iNOS antibody, clone 2A1-F8 (R&D Systems, Oxford, UK). Several antibodies to human iNOS were trialled and the antibody used was found to be the best available at the time and was fully validated. The antibody was used at 10 μg/ml in Western blotting buffer (25 mM Tris, 0.5 M NaCl, 0.05% Tween 20) at pH 7.5, containing 2% nonfat dry milk. After add-

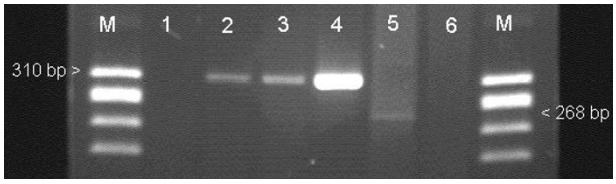

Figure 4
Induction of iNOS mRNA expression in human fetal membranes (FM), cultured without serum, following treatment with LPS (1 μg/ml) alone or in combination with sodium butyrate (SB) (2 mM). Lane 1 FM control; lane 2 FM + LPS; lane 3 FM LPS + SB; lane 4 CHOiNOS positive control; lane 5 iNOS short standard; lane 6 PCR blank (no template); lane M ΦX174 DNA/Hae III Marker (Promega). Lanes 2–4 show characteristic iNOS bands at 310 base pairs (bp). One example is shown of four similar results with four different samples of fetal membranes

ing 150 μl of blocking buffer (3% bovine serum albumin in phosphate buffered saline) the slides were incubated at 25°C for 5 minutes. The buffer was removed and 100 μl of primary antibody solution was added, followed by an overnight incubation in a humidified chamber at 2–8°C. The slides were washed twice in phosphate buffered saline, then 150 μl of a secondary antibody (horseradish peroxidase conjugate – anti-mouse, diluted 1:1000 in blocking buffer, Dako, Cambridge, UK) solution were added. The slides were incubated at 25°C for 1 hour and washed twice. 150 μl of 3,3-diaminobenzidine (DAB) substrate (1 DAB tablet in 15 ml PBS + 12 ml of 30% hydrogen peroxide) were added to the slide, and the reaction was allowed to proceed for 8 minutes. The substrate was rinsed off with distilled water and one drop of Harris Haemotoxylin was added and incubated for 1 minute. The stain was rinsed off with distilled water and one drop of Aquamount mounting medium was placed on the section before fixing the cover slide. For the negative controls the same procedure was followed, but the primary antibody was omitted. All reagents were obtained from Sigma (Poole, UK), unless otherwise stated.

Statistical analysis

Quantitative results are expressed as means ± standard deviations or standard errors, as indicated. The data were tested for normal distribution and statistical analysis was by unpaired T-test or one-way ANOVA using the Bonferroni post hoc test and statistical significance was taken as $P < 0.05$.

Results

Tissue culture

The viability of the cells comprising the membrane explants in culture was greater than 99% during the time period of the experiments, as assessed by the diaphorase staining technique and microscopy (results not shown). A time profile study showed a peak and decline in NO production within 24 hours in culture (data not shown). The fresh membranes were categorised into two groups, those which were initially active (expressing iNOS) (n = 3) and those which were initially inactive (not expressing iNOS) (n = 19), as detected by the standard RT-PCR method (Figure 1). However, very low levels of iNOS mRNA were found in all membrane samples by nested RT-PCR (Figure 2). The faint second band of a smaller size than 262 base pairs was present in all of the nested RT-PCR gels and appeared to be an artefact of nested amplification. All membrane samples produced NO in culture, as detected by the measurement of NO_2^- in the culture medium. Culturing the membrane explants with serum consistently (n = 3) induced the expression of iNOS mRNA (Figure 3), compared to the fresh membrane samples.

Effects of LPS

In the membrane samples which were initially inactive, LPS and LPS with sodium butyrate induced the expression of mRNA for iNOS, compared to control, when cultured in the absence of serum (n = 4) (Figure 4, Table 1). LPS and sodium butyrate did not significantly increase the production of NO_2^- by the membrane explants cultured without serum (n = 4) (Table 1).

Effects of PAF

PAF (1 μM) significantly increased ($P < 0.01$) the production of NO by membrane explants cultured with serum (Figure 5, Table 1). In the membrane samples which were initially inactive, PAF (10 nM – 1 μM) did not induce the expression of mRNA for iNOS, compared to control, when cultured in the absence of serum (Figure 6, Table 1) and PAF did not significantly stimulate NO production under these conditions (Table 1).

Immunohistochemistry

Immunohistochemical staining of samples of fetal membranes showed the presence of iNOS protein in four post-labour samples (Figure 7), but not in four samples obtained pre-labour (not shown). The chorion (CR) showed generalised brown staining for iNOS, also there was intense staining in the amnion epithelium (AE). In the amnion (AM), the iNOS expression was more localised. The four samples showed a similar staining pattern.

Discussion

This method for maintaining human fetal membrane discs in culture has been previously validated [5,19,36])

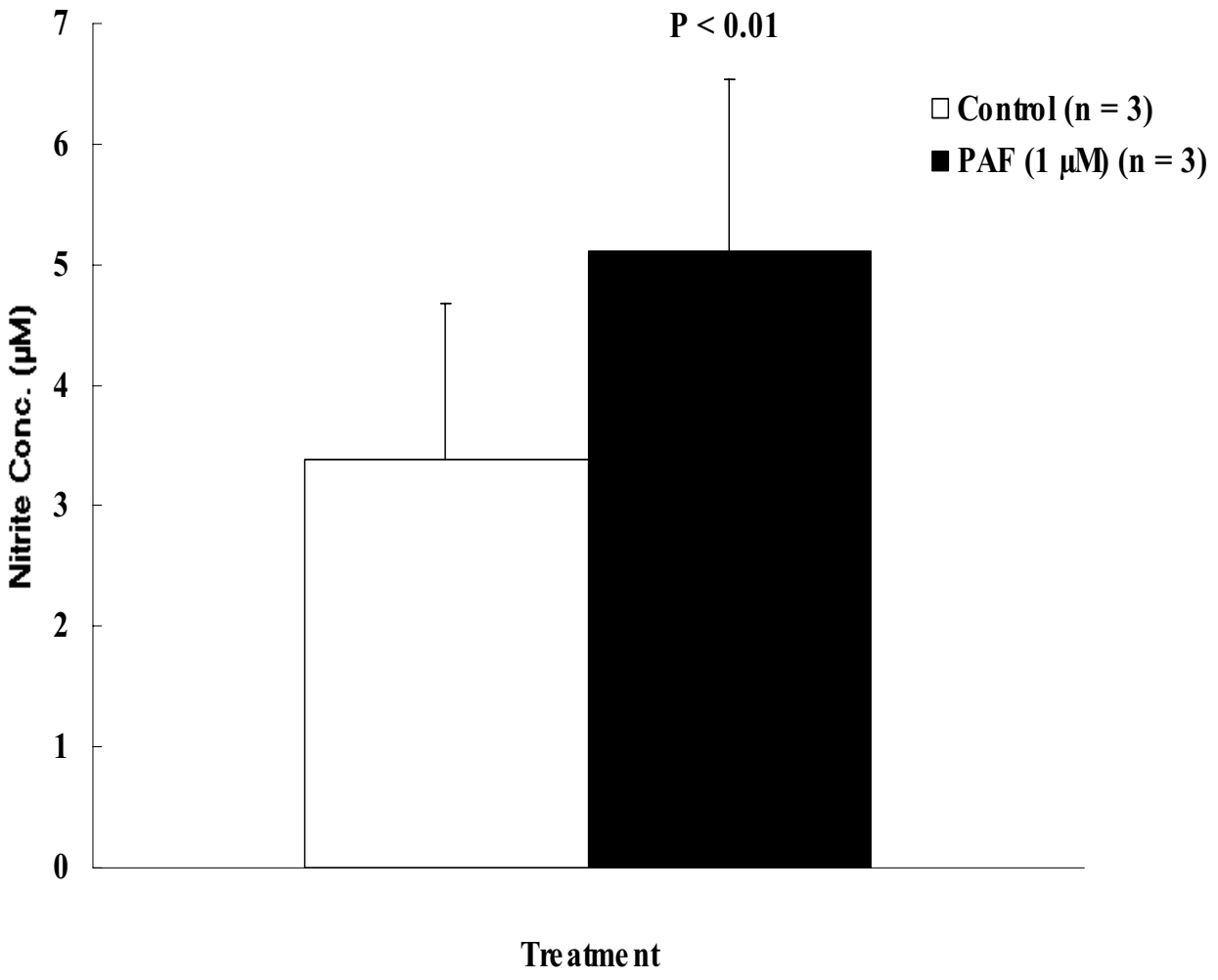

Figure 5
Mean concentrations of NO2- in medium of human fetal membrane discs cultured for 24 h with serum under control conditions and in the presence of PAF (1 μM). Mean + SEM (n = 3). All of the fetal membrane samples were initially negative for iNOS mRNA, as detected by the standard RT-PCR method.

and the categorisation of fetal membrane samples into initially active and inactive has been reported previously [37]. This study found that culturing in the presence of serum induced iNOS expression, which confirmed previous findings that culturing with serum can non-specifically induce the expression of enzymes, such as cyclooxygenase-2 [34]. Numerous growth factors and cytokines present in serum may have been responsible for this action, however Cytomix is normally required to induce iNOS expression [38]. It is possible that the fetal membranes are somehow primed for the expression of iNOS, compared to other tissues. As culturing with serum was able to induce iNOS expression, the findings from the experiments conducted in the absence of serum were perhaps more important (Table 1). In the present study, LPS was found to induce iNOS expression in the membrane explants cultured without serum which were initially inactive. In light of this novel observation, it is possible that exposure to bacterial products in utero could induce NO production by the fetal membranes. NO produced by the

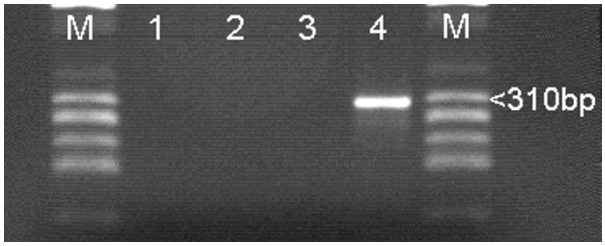

Figure 6
No induction of iNOS mRNA expression in human fetal membranes (FM), cultured without serum, following treatment with PAF (10 nM). Lane 1 fetal membranes immediately after collection, lane 2 control, lane 3 = 10 nM PAF, lane 4 positive control, lanes M ΦX174 DNA/Hae III marker. This is one example of four identical results with four different samples of fetal membranes. The fetal membrane samples were initially negative for iNOS mRNA, as detected by the standard RT-PCR method.

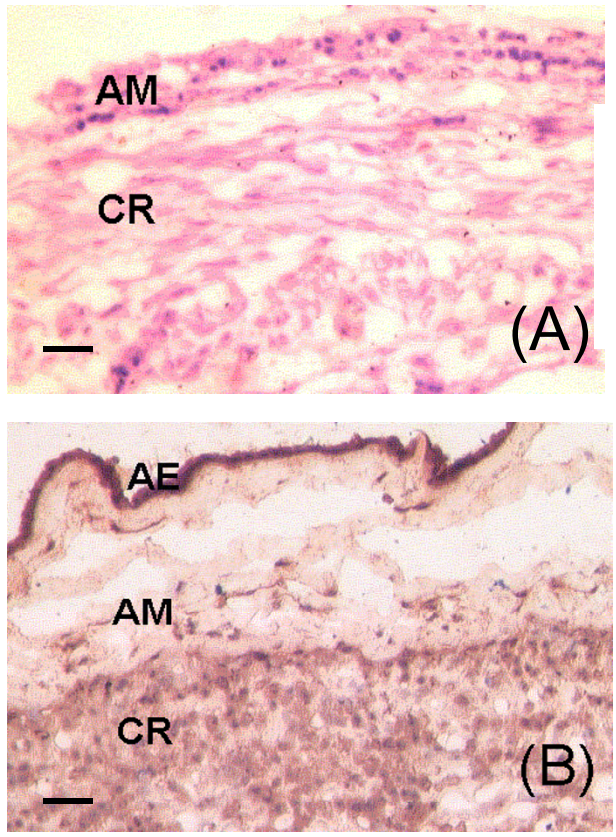

Figure 7
Detection of iNOS protein by immunohistochemical staining (B) in human fetal membrane sections obtained after vaginal delivery and (A) negative control (minus the primary antibody). CR: chorion, AE: amnion epithelium, AM: amnion. Bar = 50 μm.

membranes adjacent to the cervix could diffuse into the surrounding tissue and initiate a local inflammatory reaction, which could lead to premature rupture of the membranes. The half-life of NO (5–15 seconds under physiological conditions) is long enough for it to diffuse through several cell layers and cause subsequent effects. NO is known to be a potent inflammatory mediator [39] and to be involved in cervical ripening [24,33]. Intrauterine infection is thought to be a possible initiator of preterm labour [2-4]. It should be stressed that the pro-inflammatory role of NO is being considered here rather than its well established effect of relaxing the myometrium. There was induction of iNOS expression in fetal membranes exposed to LPS with sodium butyrate (Figure 4), a known promoter of gene expression [28] and a metabolite of anaerobic bacteria. In a pregnant murine model, administration of an inhibitor of NOS, aminoguanidine, prevented abortion induced by simultaneous administration of LPS, suggesting a role for NO production in the mechanism of LPS-induced abortion [40]. During validation of the RT-PCR method GAPDH was used as a housekeeping gene for the gels and a high level of consistency was found in amounts of total RNA and in loading the gels with similar amounts of product. Subsequently GAPDH was not included on the gels.

The cells expressing iNOS in fetal membranes are likely to be macrophages or fibroblasts [30], although in the present study iNOS appeared to be widely expressed. The membrane explants released NO (detected as NO_2^-) into the culture medium, but the basal production was not further stimulated by LPS or sodium butyrate. It is possible that the NO was produced by a different isoform of NOS, eNOS or nNOS, which were not detectable using the methods employed, although the presence of eNOS in human fetal membranes has been demonstrated previously by Dennes *et al*. [29]. It is also possible that post-transcriptional modulation of iNOS expression during the period of culture (24 h) could explain why significant differences between the production of NO under the different conditions were not found. Availability of the substrate L-arginine could have been a limiting factor, however normally cells have an adequate supply of L-arginine from metabolism, and the culture medium contained L-arginine. Previous validation experiments involving supplementing the medium with L-arginine did not produce increases in NO production, presumably due to an already adequate availability of L-arginine (unpublished observations). The membrane discs were all of the

same diameter and approximate thickness, however responses were variable, so it is possible that any significant effect of LPS or sodium butyrate on NO production may have been obscured by random variation.

In the present study, PAF significantly stimulated NO production by the membrane explants cultured with serum. However, PAF did not induce iNOS expression or stimulate NO production in the membrane explants which were initially inactive and which were cultured without serum. As PAF stimulated NO production when iNOS was expressed, but not in the absence of iNOS expression, this suggests that the mechanism of action of PAF might involve the direct stimulation of iNOS activity. The exact mechanism of action of PAF in this case is not clear, however it is known to have a variety of actions, such as the activation of GTPase, phospholipase C, D and A2, protein kinase C and tyrosine kinase [41]. Although many of these actions lead to the mobilisation of intracellular calcium ions, the activation of iNOS is calcium independent. It is possible that PAF could stimulate iNOS activity via an indirect mechanism. PAF has been shown to induce expression of primary response genes [41] and it is known to upregulate gene expression via NF-κB and a G protein-coupled pathway [42]. PAF has also been shown to activate NF-κB and enhance its DNA binding activity [43], however PAF failed to induce iNOS expression in cultured fetal membranes in this study. The target cells for PAF in the intact membranes were not identified in this study, however in a similar study Alvi et al. [44] suggested that either amnion or decidual cells were the likely targets.

It has been stated that the biochemical role of PAF in reproduction may not be attributable solely to PAF, but to other autacoids [11], which could include NO. PAF has been previously shown to stimulate NOS activity and prostaglandin synthesis in early pregnant rat uterus [45] and NO may be the intermediate between PAF and prostaglandin synthesis. The PAF antagonist WEB-2170 has been found to inhibit LPS-induced cervical ripening in rats [46], suggesting a role for PAF in this mechanism. Elovitz et al. [47] showed that PAF is an important mediator of inflammation-induced preterm birth in a mouse model. Again, the pro-inflammatory role of NO is being considered here, rather than its relaxant role.

An important finding was the lack of expression of iNOS in pre-labour fetal membranes compared to the generalised expression in post-labour samples, detected by immunohistochemical staining and RT-PCR. This suggests that iNOS is expressed during labour in fetal membranes. This confirms the previously published lack of expression of iNOS pre-labour reported by Marinoni et al. [31], but is in contrast to Kakui et al. [32], who reported similar expression of iNOS in placental tissue from women in labour and not in labour. The widespread detection of iNOS in the tissue sections was surprising. Two specific antibodies to human iNOS (R&D Systems, Abingdon, UK) were tested and one proved to be of limited use for immunohistochemical staining (data not shown), so when the experimental work was conducted the best antibody available at the time was used. In future studies blocking experiments and an inhibition assay will be performed. The induction of iNOS could be caused by inflammatory mediators, other than PAF, and cytokines released during labour. The induction of expression of iNOS could be a key event in the initiation of labour, however, this observed induction of iNOS could also be as a result of labour, rather than a cause of it.

Conclusions

This study has provided novel evidence that LPS is able to induce iNOS expression in intact human fetal membranes in vitro. If this mechanism occurs in vivo, then it could provide one possible pathway by which premature labour could occur in response to intrauterine bacterial infection. In comparison, PAF was able to stimulate NO production from intact human fetal membranes in vitro, but not via the induction of iNOS expression. PAF may have a role as a mediator in the initiation of term and preterm human parturition, via the increased local production of nitric oxide as an inflammatory mediator.

Authors' contributions

GS designed the RT-PCR techniques and carried out the experimental work. PNN and SJD gave advice on the molecular biology techniques. WNR and DJM recruited patients and organised the collection of tissue. RJC conceived and designed the study. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Jan Digby, Steve Thomas and Mark Sullivan for helpful advice and Reena Sidhu for assistance with the preparation of the manuscript.

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