A spontaneous induction of fetal membrane prostaglandin production precedes clinical labour

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Abstract

Fetal membranes from term human pregnancies produce prostaglandins, and may respond to bacterial endotoxin or interleukin-1β (IL-1β) with increased prostaglandin E2 (PGE2) production. The effects of endotoxin persisted for up to 24 h, whereas those of IL-1β were maximal 4-8 h after addition. The maximum levels of PGE2 (200-350 pg/ml) were similar in all experiments, and were independent of the stimulus used. Not all tissues responded to these stimuli; those which did not had basal levels of PGE2 production of 200-350 pg/ml, which was not further increased by endotoxin or IL-1β. The basal production from these tissues was therefore similar to the maximal production from those tissues which responded to endotoxin or IL-1β. The high basal production of PGE2 was attributed to prior in vivo activation of the membranes such that PGE2 synthesis could not be further stimulated in vitro. Overnight pretreatment with aspirin decreased basal PGE2 production from these activated membranes to <100 pg/ml/4 h during subsequent culture in aspirin-free medium. Both endotoxin and IL-1β increased PGE2 production from the activated aspirin-pretreated membranes during this culture time, but this was transient as after 12 h of culture basal PGE2 production rose to over 200 pg/ml despite aspirin pretreatment.

Introduction

The production of prostaglandins is a key step in the biochemistry of human labour (Kelly 1994). A number of studies have shown that the fetal membranes are a major source of intrauterine prostaglandins in labour (Kierse & Turnbull 1976, Skinner & Challis 1985). The amnion and decidua are the main synthetic tissues (Okazaki et al. 1981, Lopez-Bernal et al. 1987), whereas chorionic trophoblast cells have high levels of prostaglandin dehydrogenase to convert the primary prostaglandins to inactive metabolites (Sangha et al. 1994). Expression of prostaglandin pathway synthetic enzymes has been clearly demonstrated in both amnion and decidual cells (Mitchell 1987, Romero et al. 1988), with increased levels of these enzymes found in the latter stages of pregnancy (Slater et al. 1995, Trautman et al. 1996), and in association with labour (Khan et al. 1992, Fuentes et al. 1996).

High levels of the inducible isofrom of the cyclo-oxygenase enzyme (COX-2) were in particular found at the end of pregnancy and in labour (Khan et al. 1992, Slater et al. 1995, Fuentes et al. 1996, Trautman et al. 1996).

Many factors are known to increase the production of prostaglandins from amnion or decidual cells (Mitchell et al. 1990, Mitchell 1991). The mechanisms through which these effects are exerted have not been fully characterised, although many of these factors are known to regulate the expression of COX-2 (Mitchell et al. 1993, Edwin et al. 1996). Many stimuli are members of the inflammatory pathway, but digestion of tissues with enzymes is now known to activate the production of cytokines, prostaglandins and proteolytic enzymes (Kauma et al. 1992, Lonsdale et al. 1996, Qin et al. 1997). These changes in tissue biochemistry also complicate interpretation of the data obtained, as it is not clear how the biochemistry of these cells in vitro is related to their normal in vivo function.

Some studies have suggested that intrauterine prostaglandin levels increase prior to the onset of labour (Kierse et al. 1977, Mitchell et al. 1995), and high levels of COX-2 are expressed in fetal membranes prior to labour (Slater et al. 1995, Trautman et al. 1996). On this basis we predict that fetal membrane prostaglandin production may be elevated prior to clinically recognised labour. To test this hypothesis, we have determined basal PGE2 output from human fetal membranes, as well as investigated the effects of bacterial endotoxin (a model for infection-induced preterm parturition) (Mitchell et al. 1990), and IL-1β (a known stimulus) (Mitchell et al. 1993), on the production of prostaglandins in serum-free medium.

Materials and Methods

Human fetal membranes were obtained after the delivery of normal infants by elective caesarean section from pregnancies which were of 37-40 weeks gestation. Tissues were not used from patients who showed any evidence of labour, infection or pre-eclampsia. Patients had not taken any anti-inflammatory compounds for 2 weeks prior to delivery. Ethics committee approval was obtained to use tissues which would otherwise have been discarded. Immediately after delivery, the fetal membrane was cut from the placenta, and transferred to the laboratory in sterile phosphate-buffered saline containing antibiotics (1% w/v penicillin-streptomycin). The remaining stages were done under sterile conditions. Discs of fetal...
membrane were cut with a sharpened punch and incubated in Medium 199 supplemented with ITS (insulin-transferrin-selenium, containing linoleic acid and bovine serum albumin) (Croxtall et al. 1990, Rajasingam et al. 1998). Each disc (1.4 cm in diameter) was cultured overnight at 37°C in the above medium in an atmosphere of 5% CO₂ : 95% air. Fresh medium was added to the membranes prior to the initiation of any experiments.

In those experiments in which the effects of aspirin were studied, the inhibitor was present at a final concentration of 1 mM during the overnight incubation. Discs were washed and fresh medium, without aspirin, was added prior to the addition of any stimuli.

Stimuli were added to the membranes at the concentrations shown in the text, and the incubations continued for time periods ranging from 4 to 24 h. At the end of the experiments, the medium was removed from the membranes and frozen at −20°C until assay for PGE2 by ELISA (Amersham International). All culture conditions were repeated in triplicate on tissues from each membrane, and the median value from each culture condition used for further analysis. Each experiment was repeated at least 4 times, and the median data combined to give the data shown in this paper. Differences in net PGE2 output were assessed by 2-way ANOVA (Statview II, Macintosh computer).

The expression of COX-2 by fetal membranes was investigated. RNA was extracted with TRIzol and stored at −80°C until use. 1 µg of total RNA diluted into a total of 10 µl nuclease-free water and denatured at 70°C for 4 mins. To this was added 4 µl of reverse transcription buffer (Gibco), 1 µl random hexanucleotide primers (0.25 µg/ml, Pharmacia), 2 µl of dithiothreitol (100 mmol.l⁻¹), 2 µl of deoxynucleotide triphosphates (dNTPs) (10 mmol.l⁻¹) (Gibco), 0.25 µl RNase inhibitor (40 595 u/ml, Pharmacia) and 0.5 µl M-MLV reverse transcriptase (200 u/µl, Gibco). After incubation at 37°C for 1 h, the reaction was stopped by heating to 90°C for 4 mins. 80 µl of nuclease-free water was added and the products stored at −20°C.

The primers for the polymerase chain reaction (PCR) were those used previously (Slater et al. 1995). The amplification mixture for PCR was 2.5 µl buffer (10xNH₄ buffer), 0.75 µl MgCl₂ (50 mmol.l⁻¹), 0.5µ1 dNTPs (10 mmol.l⁻¹), 0.1 µl T/A polymerase (5 u/µl Biotaq), 0.25 µl each of sense and antisense primers (0.5 µg/µl) and 15.65 µl nuclease-free water. 30 µl of mineral oil was used to prevent evaporation. The PCR conditions used were denaturation at 94°C, primer annealing at 58°C and primer extension at 72°C for 30 s each. A cycle profile was established for both cDNA species, and for all tissues studied. This was done to make certain that the cycle profile was in the exponential phase, and therefore the amount of cDNA is proportional to mRNA present in the original sample. This provides a semi-quantitative comparison of mRNA levels in different samples. The cycle profiles varied between different tissues, so the numbers of PCR cycles used are shown with the figure. The products were separated on an agarose gel and visualised under UV light. Both PCR products were of the expected size.

Intact fetal membranes are composed of amnion, chorion and decidua. In preliminary studies we cut 10 µm sections from fixed, wax-embedded fetal membranes to assess the distribution of adherent decidua. All tissues showed adherent decidua. The thickness of the decidua varied between discs from the same fetal membrane, as well as between different fetal membranes. These variations were observed in discs

**Figure 1** Output of PGE2 from human fetal membrane discs. (a) Basal output (■) and endotoxin-stimulated net output (▲) were determined as described in Materials and Methods. Data are means±s.e.m. from 10 experiments. *P<0.05 vs no endotoxin. (b) Net output of PGE2 (endotoxin-basal) in 10 experiments. Different experiments are shown by different symbols. Open symbols - no overall change; closed symbols - increase in production.
which showed high and low basal PGE2 output, indicating that they are not related to the extent of fetal membrane activation.

Results

Our initial analysis of the production of PGE2 from fetal membranes showed that bacterial endotoxin increased PGE2 synthesis (Fig. 1a). There were major differences in the levels of PGE2 produced from fetal membranes from different patients, such that basal production of PGE2 ranged from 30 to 250 pg PGE2/ml/8 h. As baseline PGE2 output varied so much, we subtracted the baseline in each experiment, so as to show only the effects of endotoxin (Fig. 1b). This showed that the membranes either responded strongly to endotoxin after 8 h of culture (group I), or that there was only a limited increase in net PGE2 output (group II), indicating that there may be two groups of membranes.

The full data from group I (Fig. 2) showed that basal PGE2 output was relatively low (generally <100 pg/ml), and endotoxin increased this to 200-350 pg/ml. Combining the medians from these experiments showed that there was a 4-fold increase in net PGE2 output after incubation with endotoxin (Fig. 2).

In contrast, basal PGE2 output from the group II membranes was high (generally >100 pg/ml) (Fig. 3). The net output of PGE2 in the presence of endotoxin was similar to that in control samples (Fig. 3), and there was no significant increase in PGE2 levels.

2-Way ANOVA showed that basal PGE2 output from group I membranes was lower ($P<0.05$) than the net PGE2 output from group I membranes incubated with endotoxin as well as PGE2 output from group II membranes incubated with or without endotoxin. This applied at all three time-points investigated.

We also examined the effects of IL-1β on net PGE2 output (Fig. 4). IL-1β increased net PGE2 output only from those fetal membranes which had a low basal production, and had the greatest effect after 8 h of culture (Fig. 4). IL-1β was without effect on membranes which produced high basal levels of PGE2 at all time-points examined.
Both cyclo-oxygenase enzymes may be inactivated through acetylation by aspirin (acetyl salicylate). We therefore included aspirin (1 mM) in the overnight incubation step and determined net PGE2 output from these membranes. Basal PGE2 output was decreased by aspirin treatment (Fig. 5a) compared with the untreated level after 4 h culture in fresh medium. After 12 h culture in fresh medium basal PGE2 output was unaffected by the aspirin pretreatment. Endotoxin stimulated net PGE2 output only from those membranes pretreated with aspirin (Fig. 5a). The stimulatory effect was apparent after 4-8 h of culture, but not after 12 h. In a series of experiments (Fig. 5b), aspirin pretreatment decreased basal PGE2 output 5-fold, and significant effects of endotoxin and IL-1β were detected only in the pretreated tissues. The same tissues not pretreated with aspirin produced high basal levels of PGE2, which were not increased by endotoxin and IL-1β.

Activated fetal membranes clearly showed mRNA expression for COX-2, and the addition of IL-1β or endotoxin did not affect the expression of mRNA for COX-2 (Fig. 6a). Non-activated fetal membranes had very low COX-2 mRNA

**Figure 5** Output of PGE2 from fetal membranes. (a) Intact fetal membranes were pre-incubated overnight in medium alone (open bars) or with 1 mM aspirin (thin hatched bars, thick hatched bars), and then cultured in fresh medium alone (open bars, thin hatched bars) or with endotoxin (filled bars, thick hatched bars) for the times shown. *P<0.05 vs no endotoxin. (b) Intact fetal membranes were pre-incubated overnight in medium alone (hatched bars) or with 1 mM aspirin (open bars), and then in fresh medium for 4 h under the conditions shown. All data are means±s.e.m. (n=4). *P<0.05 vs medium alone.

**Figure 6** Expression of mRNA for COX-2 and GAPDH by fetal membranes. Upper panels, COX-2; lower panels, GAPDH. (a) Activated membrane: lane 1, after overnight culture; lane 2, 4 h culture; lane 3, 4 h with IL-1β; lane 4, 4 h with endotoxin. (b) Non-activated membrane: lane 1, after overnight culture; lane 2, 4 h culture; lane 3, 4 h with IL-1β. (c) Non-activated membrane: lane 1, 4 h culture; lane 2, 4 h endotoxin. Cycle numbers were 22 for COX-2 and 20 for GAPDH in a and b, and 30 for both in (c).
levels under control conditions, and the addition of IL-1ß or endotoxin increased the expression of mRNA of COX-2 (Fig. 6b,c). The cDNAs were of the sizes expected (305 bp for COX-2 and 598 bp for GAPDH). The intensity of the bands for GAPDH in both activated and non-activated membranes was not affected by incubation with IL-1ß or endotoxin.

Discussion

Our data support the hypothesis that increased prostaglandin production may precede the clinically detectable onset of labour, such that about 50% of the membranes examined show high basal PGE2 output (>100 pg/ml/8 h). This activation (increased basal PGE2 output) must be a persistent phenomenon, as the membranes are incubated overnight, and then for a further 24 h (a minimum of 40 h in total). No serum was present in the medium, as it is well known that serum can induce COX-2 (O’Banion et al. 1991), so it seems that an endogenous factor must activate fetal membrane PGE2 synthesis. It must be emphasised that none of the tissues were taken from women with any evidence of cervical dilation or increased myometrial contractions which could be considered as active labour. It therefore follows that increased prostaglandin production from fetal membranes precedes the clinical recognition of labour, which is consistent with the current hypotheses of human parturition (Kierse & Turnbull 1976, Skinner & Challis 1985, Kelly 1994).

This sustained activation of prostaglandin synthesis seen in some fetal membranes prior to labour implies increased COX activity. Since COX has a short half life and undergoes degradation during catalysis (Marshall et al. 1979), increased COX activity requires increased COX protein synthesis. This is mainly thought to be through increased expression of COX-2 mRNA, although stabilisation of mRNA may also be involved (Slater et al. 1994). It is COX-2 which is principally expressed in fetal membranes and whose expression increases with labour (Slater et al. 1995).

Basal COX-2 expression in fetal membranes was higher in the activated membranes (Fig. 6a) than in the non-activated membranes (Fig. 6b). The same numbers of PCR cycles were used, so the difference in cDNA band intensity between activated and non-activated tissues can be attributed to the presence of lower levels of mRNA in the tissues. We cannot tell whether this is due to increased transcription, increased mRNA stability, or a combination of these. In the activated membranes the stimuli had no effect on mRNA levels, which is consistent with the lack of effect on PGE2 output (Figs 3 and 5). In contrast, both IL-1ß and endotoxin increased COX-2 mRNA in non-activated membranes (Fig. 6b,c), which also correlates with the increased PGE2 output. These data are consistent with previous findings which implicated an induction of COX-2 in human parturition (Slater et al. 1995), and imply that this may occur before labour is clinically recognised.

In most cell types induction of COX-2 is rapid, and mRNA concentrations then fall again, so the long-term activation seen in some fetal membranes may have a different mechanism. IL-1ß increases COX-2 gene expression through activation of NFkB (Newton et al. 1997) by loss of IκB (Rice & Ernst 1993). There are two forms of the NFkB-IκB complex, namely NFkB-IκBα and NFkB-IκBβ. NFkB can induce IκBα, but not IκBβ, so dissociation of the former would lead to a transient induction of COX-2 as increasing levels of IκBα would limit the stimulation (Sun et al. 1993). In contrast, dissociation of NFkB-IκBβ would lead to a more permanent upregulation of COX-2 expression. It is not clear whether such changes would last for the 40 h or more used in this study; other mechanisms may therefore be involved, and further studies are needed to identify them.

Aspirin decreased basal net PGE2 output from activated fetal membranes. Both endotoxin and IL-1ß increased PGE2 release over this basal level for up to 8 h of culture. This is consistent with inactivation of COX enzymes by aspirin during the overnight incubation, and the synthesis of active enzymes during the following period. PGE2 can be further stimulated by inflammatory mediators during short incubations, but this is transient as the underlying decidual activation leading to labour is the dominant effect by 12 h of culture. The use of aspirin is not ideal, as this is an obvious pharmacological intervention. However, as there is no way to recognise prospectively the activated membranes, we recommend that 50% of the fetal membrane discs to be studied in each experiment are preincubated overnight with aspirin. The experimental conditions should then be duplicated in aspirin-pretreated and aspirin-untreated discs. Useful data can thus be obtained from virtually all fetal membranes, although more emphasis should be given to the information obtained from the unactivated membranes, i.e. those which show low basal prostaglandin synthesis and respond to stimuli without aspirin pretreatment (Group I). In a substantial series of experiments, these can be selected for detailed study.

Our findings are consistent with previous studies which have clearly shown that unactivated fetal membranes, or the component tissues, respond to the stimuli used in this investigation by increasing prostaglandin production (Mitchell et al. 1990, Mitchell 1991), so the results obtained in Figs 1, 2 and 4 were as expected. Furtherstudies are required to identify definitively the mechanisms through which these factors regulate prostaglandin production from intact fetal membranes, although we anticipate that induction of COX-2 is likely to be the main regulatory step.

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References

Rice NR & Ernst MK 1993 EMBO Journal 12 4685-4695.
Sun SC, Ganchi PA, Ballard DW & Greene WC 1993 Science 259 1912-1915.