Regulation of prostaglandin production in intact fetal membranes by interleukin-1 and its receptor antagonist

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Abstract

There is strong evidence for the involvement of inflammatory mediators such as interleukin (IL)-1 in the biochemical mechanisms of parturition. Therefore the effects of the IL-1 family (IL-1α (1 ng/ml), IL-1β (1 ng/ml) and the IL-1 receptor antagonist (IL-1ra) (10 ng/ml)) on the regulation of prostaglandin synthesis in term human fetal membranes were investigated. It was found that, after 4 h of culture, IL-1β increased prostaglandin E₂ (PGE₂) output approximately twofold. This was associated with both a significant increase in cyclo-oxygenase-2 (COX-2) mRNA levels (approximately fourfold compared with control) and translocation of cytoplasmic phospholipase A₂ (cPLA₂) from the cytosol to the membrane fraction. IL-1α was less effective than IL-1β at stimulating PGE₂ production through similar mechanisms.

Introduction

The biochemical signals that initiate human parturition are not completely understood. There is good evidence for prostaglandins, in particular PGE₂ and PGF₂α, being important mediators in the onset of human labour by inducing myometrial contractions (Carraher et al. 1983), ripening of the cervix (Ellwood et al. 1980) and membrane rupture (Challis & Olson 1988). Prostaglandins are produced by the amnion, chorion, decidua, myometrium and placenta (Duchesne et al. 1978, Olson & Zakar 1993). The precise regulatory mechanisms responsible for their increase at term have not been fully resolved. Many factors can increase prostaglandin production, including cytokines (Mitchell et al. 1991, 1993a, Norwitz et al. 1992, Ishihara et al. 1996) and growth factors (Kniss et al. 1992).

The first step in the synthesis of prostaglandins is the hydrolysis of arachidonic acid from cell membrane phospholipids, predominantly by the action of phospholipase A₂ (PLA₂). There are multiple forms of PLA₂ which include the cytosolic group IV (cPLA₂) (Clark et al. 1991), the secretory group (sPLA₂) (Seilhamer et al. 1989) and a cytosolic Ca²⁺-independent PLA₂ (iPLA₂) (Buhl et al. 1995). The free arachidonic acid can then be converted to the intermediates PGG₂ and PGH₂ by the action of prostaglandin H synthase (alternatively known as cyclo-oxygenase (COX)), and can then be further metabolised to prostaglandins, prostacyclin or thromboxanes. There are two isoforms of COX which are encoded by different genes (Hla et al. 1986, Hla & Neilson 1992, Takahashi et al. 1992): COX-1, which is constitutively expressed, and COX-2, which can be upregulated in response to stimuli such as cytokines and growth factors (Mitchell et al. 1993a, Albert et al. 1994, Angel et al. 1994).

Fetal membrane PLA₂ activity increases throughout gestation (Schultz et al. 1975), and total cellular cPLA₂ has also been found to be high before the onset of labour in the amnion and depleted after delivery (Skannal et al. 1997). It has also been shown that IL-1β can rapidly induce cPLA₂ protein expression and activity with a parallel increase in PGE₂ synthesis in amnion-derived WISH cells, and that this can be inhibited using a cPLA₂-specific inhibitor (Xue et al. 1995).

There is evidence for increased amnion COX enzyme activity in association with the onset of labour at term (Smieja et al. 1993, Teixeira et al. 1994) and mRNA expression of COX-2 with the onset of labour (Slater et al. 1996). The free arachidonic acid can then be converted to the intermediates PGG₂ and PGH₂ by the action of prostaglandin H synthase (alternatively known as cyclo-oxygenase (COX)), and can then be further metabolised to prostaglandins, prostacyclin or thromboxanes. There are two isoforms of COX which are encoded by different genes (Hla et al. 1986, Hla & Neilson 1992, Takahashi et al. 1992): COX-1, which is constitutively expressed, and COX-2, which can be upregulated in response to stimuli such as cytokines and growth factors (Mitchell et al. 1993a, Albert et al. 1994, Angel et al. 1994).

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et al. 1995, Hirst et al. 1995). Interleukin (IL)-1β has also been reported to increase prostaglandin synthesis in both amnion primary cells and WISH cells by induction of COX-2 mRNA and protein (Mitchell et al. 1993a, Albert et al. 1994).

IL-1 is a proinflammatory cytokine which exists in two biochemically related but distinct forms known as IL-1α and IL-1β. The third member of the family is the receptor antagonist (IL-1ra) which is generally thought to compete with IL-1α and IL-1β for the cell surface receptor without initiating signal transduction (Arend 1991). There are two types of IL-1 receptor (Sims et al. 1989, McMahan et al. 1991) but only the type 1 receptor (IL-1 R1) has been shown to transduce the IL-1 signal (Sims et al. 1993). IL-1β is found in increased levels in the amniotic fluid of women in preterm labour associated with infection (Romero et al. 1989) as well as spontaneous labour at term (Romero et al. 1990). IL-1ra has been found to prevent IL-1–induced preterm labour in mice (Romero & Tartakovsky 1992), but other workers have reported that, in cultured human decidual cells, IL-1ra can act as a partial agonist of prostaglandin production (Cole et al. 1993, Mitchell et al. 1993b).

In this study, we have used an intact fetal membrane culture system to investigate how the IL-1 family regulates prostaglandin synthesis and how their effects are modulated by the receptor antagonist.

Materials and Methods

Tissue collection and culture

Fetal membranes were collected from uncomplicated pregnancies at term (38–40 weeks gestation) after elective caesarean section in the absence of labour and infection. Patients had not taken any anti-inflammatory drugs for 2 weeks before delivery and were not suffering from pre-eclampsia. Ethics committee approval was obtained to use tissues that would normally be discarded. Tissue was washed with PBS containing 10% penicillin, streptomycin and l-glutamine (Sigma, Poole, Dorset, UK). Disks of intact tissue were cut using a sharpened punch and cultured in Medium 199 supplemented with ITS (insulin, transferrin, selenium and linoleic acid, all at 0·63 mg/ml, and BSA at 0·13 mg/ml; Sigma) in multiwell tissue culture plates for 24 h at 37 °C in an atmosphere of 5% CO₂/95% air. The medium was also supplemented with 1 mM aspirin, as we have shown that fetal membranes may be preactivated and spontaneously release high levels of PGE₂ (Brown et al. 1998). After this time the medium was changed, the tissue washed with medium containing no aspirin, and incubated with or without IL-1α, IL-1β or IL-1ra or combinations of these for time periods stated in the Results section. The tissues were snap–frozen in liquid nitrogen and stored at −80 °C. The supernatants were then removed and frozen at −20 °C until analysis for PGE₂ levels by ELISA (Amersham International, Amersham, Bucks, UK). PGE₂ levels were then expressed as a fold change relative to the control levels.

RNA extraction and reverse transcriptase (RT)-PCR

Total RNA was extracted using a standard guanidinium isothiocyanate technique (Chomczynski & Sacchi 1987) and stored at −80 °C until use. RNA samples (1 µg) were denatured at 70 °C for 5 min and cooled to 37 °C. Reverse transcription was carried out using × 1 first–strand buffer (Gibco, New York, NY, USA), 0·25 µg random hexanucleotide primers (Pharmacia, Uppsala, Sweden), 10 mM dithiothreitol, 1 mM each deoxynucleotide triphosphate (dNTP; Gibco), 1 U RNase inhibitor (Pharmacia) and 40 U Moloney murine leukaemia virus reverse transcriptase (Gibco) for 60 min at 37 °C in a reaction volume of 20 µl. The reaction was stopped by incubation at 90 °C for 4 min, and the resultant cDNA stored at −80 °C until PCR amplification.

The COX-1 and COX-2 primers for PCR have been previously reported (Slater et al. 1995). The other primers used were: cPLA₂, 5'-GAG CTG ATG TTT GCA GAT TGG GTT-3' (sense) and 5'-GTC ACT CAA AGG AGA CAG TGG ATA AGA-3' (antisense); and sPLA₂, 5'-GCT GTG TCA CTC ATG ACT GTT-3' (sense) and 5'-GGA GTA CAG CTT TGT TTA-3' (antisense). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers have been described previously (Tso et al. 1985). PCR was performed in a volume of 25 µl containing 1:20 volume of the cDNA, 1 × NH₄ buffer (Bioline, London, UK), 1·5 mM MgCl₂, 0·2 mM dNTP, 125 ng each sense and antisense primer and 0·5 U Taq polymerase (Bioline). After an initial denaturation step of 2 min at 94 °C, target cDNA was amplified by denaturing at 94 °C for 30 s, primer annealing (58 °C for GAPDH and COX-2; 55 °C for sPLA₂ and cPLA₂) for 30 s and primer extension at 72 °C for 30 s. Cycle profiles were performed as described previously to ensure that the exponential phase was used to amplify the products (Slater et al. 1995). Aliquots of the PCR products were separated by agarose gel electrophoresis and visualised under UV light.

To quantify the PCR results, 5 µl of each PCR mixture was dotted on to Hybond nylon filters. The filters were first prehybridised for 1–2 h at 65 °C followed by hybridisation overnight with the appropriate [³²P]dCTP-labelled cDNA probe at 65 °C. Excess probe was removed by washing in a series of SSC buffer washes containing 0·1% SDS from 3 × SSC down

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to 0·1 × SSC. The levels of cDNA were then determined by β-counting of the filter sections. Expression of each product was then calculated as a ratio relative to the expression of GAPDH. The ratios were then represented as a fold increase or decrease in mRNA expression.

Immunoblot analysis for protein phosphorylation of cPLA₂

Fetal membrane disks were allowed to thaw in 0·5 ml homogenisation buffer (10 mM Tris–HCl, pH 7·4, 1 mM EDTA and 1 mM sodium orthovanadate) containing the protease inhibitors pepstatin A (1 µM), phenylmethylsulphonyl fluoride (0·1 mM) and E-64 (trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane) (10 µM). Tissue was homogenised in short bursts on ice and the total lysate was then centrifuged for 10 min at 13 000 g to pellet cell debris. Protein levels were determined by Bradford assay using BSA as the standard, and 200 µg of each sample was loaded on an SDS–12% polyacrylamide gel. To demonstrate a phosphorylation-stimulated shift in cPLA₂ mobility (Lin et al. 1992a, Croxtall et al. 1996), the separating gel was made using an acrylamide:bisacrylamide ratio of 30:0·5. To improve the resolution of the protein bands, 67 mM imidazole was included in both the stacking gel and the sample buffer (Rittenhouse & Marcus 1984). Purified cPLA₂ (Genetics Institute, Cambridge, MA, USA) was also included on each gel as a positive control. The gels were run overnight until the 29 kDa molecular mass marker had run to the bottom of the gel. The proteins were then electrophoretically transferred to a nitrocellulose filter at 350 mA for 4 h in transfer buffer containing 16·5 mM Tris, 191 mM glycine and 20% methanol. The filter was blocked in PBS containing 0·1% Tween and 5% non-fat milk for 2 h at room temperature. cPLA₂ protein was detected by incubation of the filter overnight at 4 °C with a rabbit polyclonal antibody (Santa Cruz, Biotechnology Inc., Santa Cruz, CA, USA) which was diluted 1:500 in PBS-0·1% Tween containing 1% non-fat milk. This was followed by a 1 h incubation with an anti-rabbit IgG antibody labelled with peroxidase (Sigma). Immunolocalised proteins were then detected using a chemiluminescent detection system (ECL; Amersham International). The filters were also incubated with a β-actin goat polyclonal antibody (Santa Cruz) (1:2000 dilution) and then an anti-goat peroxidase-labelled antibody (Santa Cruz) (1:1000 dilution) to verify equal loading of the samples. The filters were stripped using stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62·5 mM Tris–HCl, pH 6·7) and incubated at 50 °C for 30 min with occasional agitation. The filter was then washed in PBS-0·1% Tween, blocked as before and incubated with either a COX-1 or COX-2 goat polyclonal antibody (Santa Cruz) (1:500 dilution). Human platelets were used as a positive control for COX-1, and, for COX-2, lymphocytes stimulated with lipopolysaccharide were used.

Immunoblot detection of cPLA₂ translocation from the cytosol to the membrane

Fetal membrane disks were homogenised in 0·5 ml translocation buffer (25 mM Hepes, pH 7·4, 5 mM EDTA, 50 mM NaF and 1 mM sodium orthovanadate). The total lysate was then spun for 10 min at 4 °C at 13 000 g, and the resulting cytosol spun at 100 000 g for 1 h at 4 °C. This gave cytosolic (supernatant) and membrane (pellet) fractions. The membrane pellet was resuspended in 40 µl translocation buffer. Then 100 µg of the cytosolic fractions and each membrane fraction were loaded on an SDS–12% polyacrylamide gel. The proteins were electroblotted, and cPLA₂ and β-actin were immunodetected as described above.

Analysis of immunoblots

The autoradiographs of each blot were scanned by a UMAX Mirage D-16L scanner and the integrated density...
of each band determined using the software package Whole Band Analyser (Genomic Solutions Ltd, Eaton Socon, Cambs., UK). The integrated density for cPLA2 or COX was then expressed relative to the density of \( \beta \)-actin for each sample.

**Statistical analysis**

The results are shown as mean ± S.E.M. For statistical analysis, the results were log-transformed and investigated by ANOVA with post-hoc analysis by Fisher’s exact test. Significance was set at \( P<0.05 \).

**Results**

We determined the optimum concentration of IL-1\( \beta \) and IL-1\( \alpha \) for stimulating PGE\( _2 \) production to be 1 ng/ml, and that for IL-1ra was 10 ng/ml. IL-1\( \beta \) significantly increased PGE\( _2 \) output from fetal membranes over twofold compared with control levels after 4 h of treatment, whereas IL-1\( \alpha \) was less potent (Fig. 1). IL-1ra alone did not increase prostaglandin release. When IL-1\( \beta \) and IL-1ra were incubated together, PGE\( _2 \) production was increased more than with IL-1\( \beta \) alone. The combination of IL-1\( \alpha \) and IL-1ra caused the same increase in PGE\( _2 \) output as IL-1\( \beta \) alone.

A similar pattern was found when the levels of COX-2 protein were investigated by immunoblotting (Fig. 2). There was a significant increase with IL-1\( \beta \) plus IL-1ra and also with IL-1\( \alpha \) treatment on its own. Treatment with IL-1\( \alpha \) or IL-1\( \alpha \) plus IL-1ra did not significantly increase the levels of COX-2 protein. Immunoblots were also performed for the COX-1 isoform. However, only very low levels of COX-1 could be detected which were not affected by any of the treatments (results not shown).

Expression of COX-1 and COX-2 mRNA was investigated using RT-PCR. COX-1 cDNA was detected in very low amounts using high cycle numbers (data not shown). As expected, COX-2 was highly abundant in fetal membranes incubated with IL-1\( \beta \), a significant fourfold increase in mRNA levels over those in controls being observed after 4 h of treatment (Fig. 3). Once again, IL-1\( \alpha \)

**Figure 2** Changes in COX-2 protein levels after treatment with IL-1\( \beta \), IL-1\( \alpha \) or IL-1ra or combinations of these treatments for 4 h: (A) typical immunoblot; (B) histogramic summary of three such immunoblots analysed by densitometry showing the mean ± S.E.M. COX-2 levels in each sample were corrected for \( \beta \)-actin concentration and then expressed as fold change compared with the control level. *Statistically significant difference (\( P<0.05 \)).
was less potent, with a trend towards an increase in COX-2 mRNA expression that was not significant. IL-1ra did not increase COX-2 mRNA expression and reduced IL-1β-upregulated COX-2 mRNA by half.

Expression of cPLA₂ mRNA after 4 h of treatment was investigated by RT-PCR (Fig. 4). This was found to be abundant but no significant changes were found with any of the treatment groups relative to control levels. Longer treatments with IL-1β for time periods of up to 72 h similarly did not result in an increase in cPLA₂ mRNA expression (data not shown). Expression of sPLA₂ mRNA was also investigated; only very low levels could be detected after high PCR cycle numbers (data not shown).

Phosphorylation of cPLA₂ reduces its electrophoretic mobility, which offers a convenient way of following agonist-induced changes in the phosphorylated state. Possible changes in the phosphorylated state of cPLA₂ after the various treatments were therefore investigated (Fig. 5). IL-1β, IL-1α, IL-1ra and IL-1β plus IL-1ra treatment all significantly increased the amount of phosphorylated protein but did not change the ratio of the phosphorylated to unphosphorylated form.

Translocation of cPLA₂ from the cytosol to the endoplasmic reticulum (ER) and nuclear membranes after 2 h of each of the different treatments was also investigated (Fig. 6A–C). IL-1β and IL-1α produced a significant increase in cPLA₂ translocation from the cytosol to the membrane. Treatment with IL-1ra alone and IL-1β plus IL-1ra resulted in an approximately equal distribution between the cytosol and membrane and an overall significant increase in the total mass of cPLA₂ (Fig. 6D).

Discussion

This study examined the mechanisms by which IL-1α, IL-1β and IL-1ra regulate prostaglandin biosynthesis in intact fetal membranes collected at term. The tissue explants used consisted of adherent amnion, chorion and decidua, which is a better representation of in vivo conditions than isolated cell types. IL-1β was found to be a potent stimulator of PGE₂ production from intact fetal membranes, which is consistent with studies performed using amnion and decidual cells (Mitchell et al. 1993a, Albert et al. 1994, Cole et al. 1995, Kennard et al. 1995, Mitchell et al. 1993b, Albert et al. 1994, Cole et al. 1995, Kennard et al. 1995, Mitchell et al. 1993b, Albert et al. 1994, Cole et al. 1995, Kennard et al. 1995).
Xue et al. (1995). This increase in PGE$_2$ production was associated with a large increase in COX-2 mRNA expression (Fig. 3). The increase in COX-2 protein was less dramatic than that of COX-2 mRNA (Fig. 2). This is probably due to the instability of COX-2 protein, which has a half-life of approximately 30 min (DeWitte & Meade 1993, Evett et al. 1993). COX-2 mRNA is also unstable (DeWitte & Meade 1993), and IL-1$\alpha$ may cause upregulation of its expression to compensate for this. IL-1ra reduced IL-1$\alpha$-stimulated COX-2 mRNA expression by half, which almost reached significance ($P=0.083$), suggesting that it antagonises the effects of IL-1$\beta$ at the mRNA level. Other workers have found that IL-1ra suppressed IL-1$\alpha$-driven prostaglandin synthesis in human endometrial cells by inhibiting the induction of COX-2 mRNA and protein (Kniss et al. 1997). This group also found that when endometrial cells were incubated with IL-1ra at a dose of 10 ng/ml, prostaglandin production was significantly higher than the control, suggesting that IL-1ra may have partial agonist-like properties.

cPLA$_2$ is selective for phospholipids with arachidonic acid in the sn-2 position and has been shown to be coupled to the hormonal release of arachidonic acid (Lin et al. 1992a), and the accumulation of cPLA$_2$ has been reported after IL-1 treatment (Lin et al. 1992b, Angel et al. 1994, Xue et al. 1995). It is therefore probably involved in receptor-mediated eicosanoid production. Two mechanisms are involved in the activation of cPLA$_2$: it may be post-translationally modified by phosphorylation, which increases enzyme activity, and this is followed by Ca$^{2+}$-dependent translocation to the nuclear membranes.

None of the IL-1 family was found to change the expression of cPLA$_2$ transcript in the time period studied. Longer incubations with IL-1$\beta$ of up to 72 h were also performed but no increases in cPLA$_2$ mRNA were found. Therefore post-transcriptional changes in cPLA$_2$ may be responsible for the increases in PGE$_2$ production found. In our study of the activation of cPLA$_2$ by phosphorylation and Ca$^{2+}$-mediated translocation to the nuclear envelope and/or ER, increased levels of the phosphorylated form of cPLA$_2$ were found after treatment with IL-1$\beta$, IL-1$\alpha$, IL-1ra and IL-1$\beta$ plus IL-1ra. This was a result of increased cPLA$_2$ protein mass rather than a change in the ratio of phosphorylated to unphosphorylated protein and

Figure 6 (A) Immunoblot of cytosolic and membrane fractions following treatment with IL-1$\beta$ or IL-1ra. Lane 1 is a cPLA$_2$ positive control. (B) Immunoblot of cytosolic and membrane fractions following treatment with IL-1$\alpha$ or IL-1ra. (C) Analysis of three immunoblots by densitometry showing the percentage distribution of total cPLA$_2$ in either the cytosolic or membrane fractions after 2 h of each of the treatments. (D) Change in total cPLA$_2$ levels (combining those found in the cytosolic and membrane fractions). Results shown are the integrated density (mean $\pm$ S.E.M) where cPLA$_2$ levels were corrected for $\beta$-actin concentration and expressed as fold change compared with the control. *Statistically significant difference ($P<0.05$).
therefore a higher level of active protein compared with the control. This induction by IL-1β of cPLA₂ protein levels without a change in the phosphorylation status of the enzyme has also been reported in amnion-derived WISH cells (Honican et al. 1998).

IL-1β increased translocation of cPLA₂ to the membrane compared with the control. As Ca²⁺ appears to be essential for cPLA₂ activation, IL-1 may stimulate the production of a Ca²⁺-mobilising agent (Ikeda et al. 1990). Translocation was inhibited by IL-1ra, with equal distribution in the cytosol and membrane fractions. However, IL-1ra treatment did lead to an increase in cPLA₂ mass compared with treatment with IL-1β alone. This may be the mechanism by which IL-1ra enhances the effect of IL-1β, i.e. it increases cPLA₂ mass which can then mobilise more substrate to be metabolised by COX to give PGE₂. Unfortunately, the assay used for cPLA₂, which measured the release of radio-labelled arachidonic acid from arachidonic-containing phospholipid, was not sensitive enough to give accurate measurements in the tissue explants.

COX-1 and sPLA₂ transcripts were virtually undetectable after treatment with IL-1β, IL-1α or IL-1ra and are therefore probably not involved in their regulatory mechanism. A similar finding has been reported for the treatment of human synovial cells with IL-1 (Angel et al. 1994).

In conclusion, IL-1β activates PGE₂ output from fetal membranes by upregulating COX-2 gene expression and increasing the translocation of cPLA₂ from the cytosol to the cellular membranes. IL-1α is a less potent activator of PGE₂ output by similar mechanisms. A very important finding was that IL-1ra can upregulate PGE₂ production from IL-1β-stimulated fetal membrane explants, and this appeared to be as a result of increasing cPLA₂ protein levels. As the transcript levels do not change, it suggests that IL-1ra may stabilise either the mRNA or the protein. It has been postulated that IL-1ra may bind to the decoy type II IL-1 receptor, thereby dislodging any bound IL-1, which could then in turn bind to the type I receptor (Kniss et al. 1997).

However, the results of our study suggest that IL-1ra has different effects on the prostaglandin pathway than those of IL-1β; it acts on cPLA₂ protein levels, whereas IL-1β acts primarily by upregulating COX-2. IL-1ra has been found to block IL-1-induced prostaglandin production by amnion and chorion primary cells (Romero et al. 1992b) and so it would be appear that IL-1ra has a unique role in the decidua. The signal transduction pathway by which IL-1ra achieves this effect is beyond the scope of this paper and needs to be addressed in future work.

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