Corticotrophin-releasing hormone and platelet-activating factor induce transcription of the type-2 cyclo-oxygenase gene in human fetal membranes

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Corticotrophin-releasing hormone (CRH) and platelet-activating factor (PAF) are considered to be involved in the physiological processes of human labour. Both may have dual effects, directly regulating myometrial contractility and fetal membrane prostaglandin production. During this study, we investigated the mechanisms through which CRH and PAF exert their latter effect. CRH and PAF increased prostaglandin production from intact fetal membrane discs, with a maximum stimulation after 8 h of culture. Reverse transcription–polymerase chain reaction (RT–PCR) analyses using primers specific for type-2 cyclo-oxygenase (COX-2) showed that CRH and PAF increased the transcription of COX-2 mRNA two-fold after 8 h culture. These data indicate that the increased fetal membrane prostaglandin production in response to CRH or PAF may involve the induction of COX-2.

Key words: COX-2 expression/corticotrophin-releasing hormone/fetal membranes/platelet-activating factor/prostaglandin E₂

Introduction

An inflammatory mechanism has been proposed to be involved in labour at all gestational ages (Lopez Bernal et al., 1993; Dudley et al., 1996), but it is not clear what the main physiological factors which initiate this process are. A number of intrauterine products, including corticotrophin-releasing hormone (CRH) and platelet-activating factor (PAF) have been implicated in the process of labour. A role for CRH in labour is suggested by the marked increase in free CRH in the maternal circulation in the last 3 weeks of pregnancy (Ellis et al., 1988; McLean et al., 1995). CRH may influence the progression to labour through two different mechanisms. Firstly there may be a direct increase in myometrial contractility at term (Quartero et al., 1991), through increased expression of a high-affinity receptor for CRH prior to labour (Hillhouse et al., 1993) which seems to be linked to a G regulatory protein (Grammatopoulos et al., 1994). In addition to the effects on muscular contractility, CRH stimulates the production of prostaglandins from human decidua (Jones and Challis, 1989; Petraglia et al., 1995), which may be an additional pathway through which it causes labour. Decidua is thought to be a major source of the prostaglandins which initiate and sustain the process of parturition (Roseblade et al., 1990; Kelly, 1994). On the basis of these observations, it is considered that CRH could be a placental signal for human parturition.

PAF is produced from the fetal lungs (Frenkel et al., 1996). Lung maturity is a critical factor in the survival of preterm infants, and PAF released from the maturing lungs could be a fetal signal to initiate labour (Toyoshima et al., 1995). Increased concentrations of PAF are present in the amniotic fluid in both preterm and term labour (Hoffman et al., 1990), indicating that maturing fetal lungs at term are not the only source. PAF production is increased in the inflammatory response in non-uterine tissues, so a general intrauterine inflammatory reaction (as in preterm labour) could also contribute to increased concentrations of PAF. It seems likely that PAF will regulate more than one intrauterine response, as it can directly regulate uterine contractility (Kim et al., 1995) through myometrial receptors, as well as stimulate the production of prostaglandins from fetal membranes (Morris et al., 1992).

In most intrauterine tissues it is now apparent that the main regulation of prostaglandin production is through the induction of the type-2 cyclo-oxygenase enzyme (COX-2) (Williams and DuBois, 1996). The effects of CRH on the expression of this enzyme in intact human fetal membranes were therefore examined. CRH has previously been reported to increase prostaglandin synthesis from isolated human decidual cells (Jones and Challis, 1989), but recent studies have indicated that the dissociation of tissues including human decidua can induce the spontaneous production of cytokines and prostaglandins (Kauma et al., 1992; Lonsdale et al., 1996; Qin et al., 1997). Such preparations may therefore not reflect normal physiological functions, and tissue explants are better models in which to study the control of prostaglandin synthesis (Kauma et al., 1992; Lonsdale et al., 1996), although it is clear that CRH may increase the production of prostaglandins from isolated cells (Jones and Challis, 1989). Therefore, in this study we used fetal membrane explants (comprising amnion, chorion and decidua) which retain the full integrity of the tissues. This model has been described previously (Brown et al., 1998; Rajasingam et al., 1998).
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 with local ethics committee approval. 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. Immediately after delivery, the fetal membrane was cut from the placenta, and transferred to the laboratory in sterile phosphate-buffered saline (PBS) containing antibiotics (1% v/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 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.

Different concentrations of CRH (1–100 nM) or PAF (100 pM to 1 µM) were added to the membranes, and the incubations continued for time periods ranging from 4–24 hours. At the end of the experiments, the medium was removed from the membranes and frozen at –20°C until assay for prostaglandin E₂ (PGE₂) by enzyme-linked immunosorbent assay (ELISA) (Amersham Pharmacia Biotech). The greatest increases in PGE₂ production were observed in the presence of 100 nM CRH or 10 nM PAF, so these concentrations were used in all experiments. All culture conditions were repeated in triplicate on tissues from each membrane, and this was repeated on at least three different fetal membranes for the dose–response studies. A mixed model analysis of variance was used to determine the effects of CRH or PAF on PGE₂ production in 11 separate replicate experiments to assess changes in PGE₂ production between 4 and 12 h. The distribution of the data was tested for normality with the Watson statistic, and Bartlett’s test was used to check for equal variances. The data are presented as mean changes in PGE₂ production compared with control values (medium alone) with 95% confidence intervals and significance values. The latter have been adjusted using the Bonferroni correction to account for multiplicity. Not all tissues responded to CRH or PAF, but many of these had high basal PGE₂ production, and pre-activation of the tissues (Brown et al., 1998) may explain these data. All other materials from Sigma.

Membrane discs were snap-frozen in liquid nitrogen and stored at –70°C. It has previously been shown that human fetal membranes may be activated and spontaneously release high concentrations of PGE₂ prior to the onset of labour (Brown et al., 1998). All membranes selected for further analysis in this study were of the non-activated type. Membrane discs from three separate experiments (i.e. from three different patients) were selected for analysis of mRNA by reverse transcription and polymerase chain reaction on the basis of their PGE₂ production in response to CRH or PAF. They were homogenized immediately after the addition of TRIzol reagent (Molecular Research Centre Inc.), and the RNA extracted. 1 µg of RNA was reverse transcribed and used as a template for the polymerase chain reaction (PCR). Cycle profiles for COX-2 and glyceraldehyde phosphate dehydrogenase (GAPDH) were generated by PCR with specific primers (Slater et al., 1995), to ensure that the linear part of the PCR profile was used, and that semi-quantitative comparisons could be obtained. 28–32 cycles (for COX-2 and GAPDH) were found to be appropriate for all the samples investigated. 5 µl of each PCR reaction was dotted onto Hybond nylon membranes (Amersham Pharmacia Biotech). These were then washed in denaturing solution (1.5 M NaCl, 0.5 M NaOH), then in neutralizing solution (1.5 M NaCl, 0.5 M Tris·HCl pH 7.5) (all from Sigma), and finally in 3x sodium chloride/sodium citrate (SSC) buffer, all other molecular materials from Gibco, except for RNase inhibitor and M-MLV (Pharmacia). The DNA was fixed to the membrane by UV cross-linking. The blots were pre-hybridized at 65°C for 1–2 h and then hybridized with radiolabelled cDNA probes for COX-2 or GAPDH at 65°C overnight. The filters were then washed with buffers of increasing stringency to 0.1× SSC, and then exposed to autoradiographic film. The levels of COX-2 and GAPDH were then quantified by scintillation counting. The ratio of COX-2 expression : GAPDH expression was determined for all samples. This ratio for CRH or PAF treated samples was then compared with the corresponding control samples, and expressed as a fold increase.

Results

Dose–response experiments indicated that 100 nM CRH was appropriate to stimulate PGE₂ production from human fetal membranes (Figure 1a), a figure consistent with previous data on human decidual cells (Petraglia et al., 1995). 10 nM PAF increased PGE₂ production from fetal membranes (Figure 1b) but both higher (Figure 1b) and lower concentrations had no effect on PGE₂ output (data not shown). All data in Figure 1 are from individual rather than combined experiments, as the
wide variability of fetal membrane responses made combining the results difficult. In the remaining experiments, CRH was used at 100 nM and PAF at 10 nM. CRH increased the output of PGE2 from intact fetal membranes (Figure 2) 4 h after addition, with maximum effect after 8 h. PAF had more variable effects at the shorter incubation time (the increase was not statistically significant), and increased PGE2 output after 8 h (Figure 2). Longer incubations (12 h) with either stimulus were less effective.

Separation of PCR products on agarose gels showed that the products generated by specific primers for COX-2 and for GAPDH were of the expected size (Figure 3). Visual examination suggested that both CRH and PAF increased the expression of COX-2 after 4 h and 8 h of culture (Figure 3), without affecting the expression of GAPDH. PCR cycle profiles for COX-2 (Figure 4) and GAPDH (not shown) were set up to identify appropriate cycle numbers for quantification; 32 cycles for COX-2 were used in the experiment shown. A semi-quantitative estimate of the expression of COX-2 cDNA was obtained by dot-blotting and hybridization as described in the methods section. This was normalized to the expression of cDNA for GAPDH from the same samples. CRH and PAF had similar stimulatory effects on COX-2 expression (Figure 5).

**Discussion**

It is likely that the fundamental mechanisms controlling the switch from the pro-pregnancy to the pro-labour state is fetal in origin and has some relationship to fetoplacental size or maturity. Both McLean et al. (1995) and Challis et al. (1995) have suggested that the timing of labour is mediated through placental release of corticotrophin releasing hormone (CRH) whose concentration in maternal plasma begins to rise ~90 days prior to the onset of labour. At 20 days prior to delivery, i.e. at ~37 weeks in the average pregnancy, CRH concentrations began to exceed concentrations of the CRH binding protein. The prelabour increase in COX-2 expression in amnion and chorion-decidua (Slater et al., 1997) mirrors that of maternal CRH (McLean et al., 1995). CRH stimulates prostaglandin E2 (PGE2) from intact fetal membranes incubated with medium alone (□) or in the presence of corticotrophin-releasing hormone (CRH) (100 nM) (■) or platelet-activating factor (PAF) (10 nM) (□). All data are means ± SEM (n = 5). *P < 0.05 compared with control.

**Figure 2.** Increase in the production of prostaglandin E2 (PGE2) from intact fetal membranes incubated with medium alone (□) or in the presence of corticotrophin-releasing hormone (CRH) (100 nM) (■) or platelet-activating factor (PAF) (10 nM) (□). All data are means ± SEM (n = 5). *P < 0.05 compared with control.

**Figure 3.** Agarose gel showing cDNA bands corresponding to type-2 cyclo-oxygenase (COX-2) and glyceraldehyde phosphate dehydrogenase (GAPDH).

**Figure 4.** Agarose gel showing cDNA bands from cycle profile for type-2 cyclo-oxygenase (COX-2).

**Figure 5.** Expression of type-2 cyclo-oxygenase (COX-2) determined by semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR), normalized to the expression of glyceraldehyde phosphate dehydrogenase (GAPDH). Expression after 8 h culture with corticotrophin-releasing hormone (CRH) (■) or platelet-activating factor (PAF) (□) is compared with culture in medium alone (□). Data are means ± SEM from three representative experiments. *P < 0.05 compared with control.
synthesis in fetal membranes (Jones and Challis, 1989) and in cultured decidua (Petraglia et al., 1995), and the findings in this paper show that this is associated with induction of COX-2. The CRH-R1 receptor is expressed on human endometrial stromal cells in culture (Di Blasio et al., 1997), and the CRH-R1α and CRH R1-c splice variants are present in fetal membranes (Karteris et al., 1998), but the target cells in intact fetal membranes have not been identified.

PAF is known to be present in amniotic fluid (Hoffman et al., 1990), and it might be expected that the amnion would be the primary target as these cells express a PAF receptor (V.C.Allport and P.R.Bennett, unpublished observations). However, the in-vitro addition of PAF to fetal membranes had only limited effects on PGE2 levels on the amnion side of the membrane (Morris et al., 1992), and the main increase in prostaglandin production was on the maternal (decidual) side (Morris et al., 1992). This suggests that decidual cells may be the primary target for PAF and further studies are needed to identify the targets for PAF within the intact fetal membrane system.

Several factors, including interleukin (IL)-1β, CRH and PAF, increase in concentration within the uterus near to term and stimulate prostaglandin synthesis through up-regulation of COX-2 expression. The CRH receptor is linked to G-protein adenyl cyclase second messenger systems (Chida and Voelkel, 1996; Han et al., 1996), and the 5’ regulatory region of COX-2 contains a cAMP-response element (CRE) (Herschman et al., 1995). Both up-regulation and down-regulation of COX-2 by cAMP has been shown in various cell types (Chida and Voelkel, 1996; Han et al., 1996; Pang and Hoult, 1997). PAF and IL-1β act to up regulate expression through the transcription factor NFkB (Kravchenko et al., 1995), and the COX-2 promoter contains two NFkB-binding elements (Newton et al., 1997). In bronchial epithelium-derived A549 cells IL-1β up-regulates COX-2 expression by activation of the RelA/NFkB1 dimer (Newton et al., 1996, 1997). Because of its central role in parturition an understanding of how COX-2 expression is controlled within the uterus should lead to better understanding of the endocrine and paracrine signals controlling the length of human pregnancy and the onset of labour.

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