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Implication of mRNA Binding Proteins in the Regulation of Cyclo-oxygenase in Human Amnion at Term

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The onset of labour is associated with an increase in prostaglandin synthesis in amnion which appears to be mediated at least in part by an increase in cyclo-oxygenase (COX) expression. We have tested the hypothesis that COX expression is controlled in amnion by the binding of a protein to the COX mRNA which may inhibit its translation, as has been seen in vascular smooth muscle cells. Using differential RNA extraction protocols which extract either total mRNA or only mRNA which is not protein bound, we have found that, in amnion, the increase in COX-2 expression seen after the onset of labour is entirely in the protein bound fraction. Unlike in vascular smooth muscle, increased expression of COX-2 in amnion cells is therefore associated with increased rather than decreased protein binding to the mRNA. This protein may be involved in translation initiation or elongation or in mRNA stability. There does not appear to be a protein bound COX-1 mRNA fraction.

It is well established that prostaglandins play a central role in the initiation and/or maintanence of human labour and that the fetal membranes, in particular the amnion, contain large stores of arachidonic acid from which prostaglandins can be synthesised (1,2). However, the mechanisms that control arachidonic acid metabolism within the fetal membranes, and which stimulate prostaglandin production at term are still poorly understood.

The initial step in the synthesis of prostaglandins is the release, by the action of phospholipases, of free arachidonic acid. It has been suggested that increased prostaglandin synthesis in the fetal membranes in association with labour is due to an increase in availability of arachidonic acid through increased phospholipase activity (3). It has also been shown that there is an increase in activity of the cyclo-oxygenase enzyme in amnion cells associated with labour at term (4). This increase and the short half life of the enzyme would suggest that cyclo-oxygenase may also be a principle factor controlling prostaglandin synthesis at term (5). Two human cyclo-oxygenase enzymes have been described. The first, cyclo-oxygenase type-1 (COX-1) is thought to represent

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a constitutive form (6), whereas the more recently isolated cyclo-oxygenase type-2 (COX-2) gene appears to represent an inducible form of the enzyme (7).

Bailey *et al.* (1988) have shown, by Northern analysis, that in vascular smooth muscle cell culture, addition of dexamethasone results in the levels of hybridisable cyclo-oxygenase mRNA being reduced by over 90% (8). They suggested that the mRNA was being converted into a nontranslated ('cryptic') form, by the binding of a glucocorticoid-induced protein. Investigation of this phenomenon used two differential RNA extraction techniques. The first (method A) uses a cationic detergent (sodium N-lauryl sarcosine) within the homogenisation buffer to optimise isolation of the glucocorticoid-suppressed mRNA present in the cell, which Bailey termed 'cryptic' mRNA. The second (method I) optimises the isolation of normal 'free' mRNA (9).

To determine whether the changes in COX expression, which have previously been seen in amnion associated with labour onset (10), are controlled by the binding of a protein to the COX mRNA we have used the differential mRNA isolation methods described by Bailey (9). Reverse transcription polymerase chain reaction (RT-PCR) was then employed to quantify levels of COX-2 and COX-1 expression from amnion collected before and after the spontaneous onset of labour.

MATERIALS AND METHODS

Intact human fetal membranes were obtained from 8 term pregnancies before the onset of labour at elective caesarean section and 8 following spontaneous vaginal delivery. Tissues were immediately rinsed in phosphate buffered saline (PBS) to remove excess blood. The amnion was separated from the chorion, snap frozen in liquid nitrogen and stored at -80°C until required. Samples were split in two and RNA extracted by both method A and method I isolation procedures.

For the method A RNA extraction, tissue was first homogenised in buffer A (4.5 M guanidine isothiocyanate, 25 mM sodium acetate, (pH 7), 100 mM 2-mercaptoethanol, 0.4% vanadyl ribonucleoside complex and 0.7% N-laurylsarcosine), and for method I RNA extraction, tissue was homogenised in buffer I (4M guanidine isothiocyanate, 0.25M sodium acetate (pH 6), 100mM 2-mercaptoethanol). The homogenates were clarified by brief centrifugation at 10,000g, 4°C to remove cellular debris. The resulting supernatants were then layered over a 5M cesium chloride cushion and centrifuged at 100,000g for 24 hours, 20°C. The RNA pellet was rinsed in 70% ethanol and resuspended in 0.3 M sodium acetate (pH 7 for buffer A homogenates and pH 6 for buffer I). The RNA was precipitated overnight with 2.5 volumes ethanol at -20°C. The RNA was pelleted and resuspended in 1mM EDTA.

For reverse transcription RNA samples of 1μg were denatured at 70°C for 5 minutes before cooling to 37°C. Reverse transcription was performed at 37°C for 60 minutes containing 0.2μg random hexanucleotide primers, and 40 units of M-MLV reverse transcriptase. The reaction was stopped by heating at 95°C for 5 minutes. A 1/20 volume of the RT reaction was used for PCR amplification. PCR amplification, from the reverse transcribed cDNA,was performed using primers specific for either COX-1 (11),COX-2 (7) or GAPDH (11). The PCR contained 1.5mM magnesium chloride, 0.2mM dNTPs, 0.25μg of each primer, and 1 unit of Biotaq polymerase (Biotaq). Reaction cycles were as follows: 94°C for 1 minute, 58°C for 1 minute 20 seconds, 72°C for 1 minute. At 22, 25 and 32 cycles of amplification, the amount of the amplified product is linear with respect to the input RNA for GAPDH, COX-2 and COX-1 primers in amnion respectively (data not shown).

Aliquots of the PCR reactions (10μl) were separated on 1.2% agarose gels and used for Southern analysis according to standard procedures (12). Filters were hybridised overnight at 65°C with either the cDNA for human COX-2, COX-1, or GAPDH. Filters were high stringency washed and exposed to autoradiographic film. Quantitation of amplified DNA was performed by densitometric analysis of autoradiographs (Biorad Model 620 densitometer). COX-2 and COX-1 expression was

normalised with respect to the expression of GADPH in parallel samples. Comparison of the expression of cyclo-oxygenase with that of the GAPDH standard allows relative changes in cyclo-oxygenase expression to be assessed. Expression is assessed as an optical density ratio of cyclo-oxygenase to GAPDH. Statistical analysis was performed using ANOVA.

RESULTS

There were no significant differences observed in the expression of COX-2 mRNA in amnion isolated by either method A or method I before the onset of labour (ie tissue from elective caesarean sections). Following spontaneous labour delivery a significant 2 fold increase in COX-2 expression was seen in amnion from RNA isolated by method A compared to that isolated by method I. No significant difference in COX-2 expression was observed when comparing RNA extracted by method I from either pre or post labour amnion (Fig. 1). There were no significant difference observed in the expression of COX-1 RNA from amnion collected either before and after labour onset. When comparing RNA samples isolated using either method A or I, no significant difference in the expression levels of COX-1 were observed (Fig.2).

DISCUSSION

DeWitt (1991) put forward the suggestion that Cox activity is regulated in two ways; firstly by controlling the rate of enzyme turnover, and secondly by a general increase in level of production of the enzyme (13). Studies have previously shown that both enzyme activity (Vmax) and COX-2

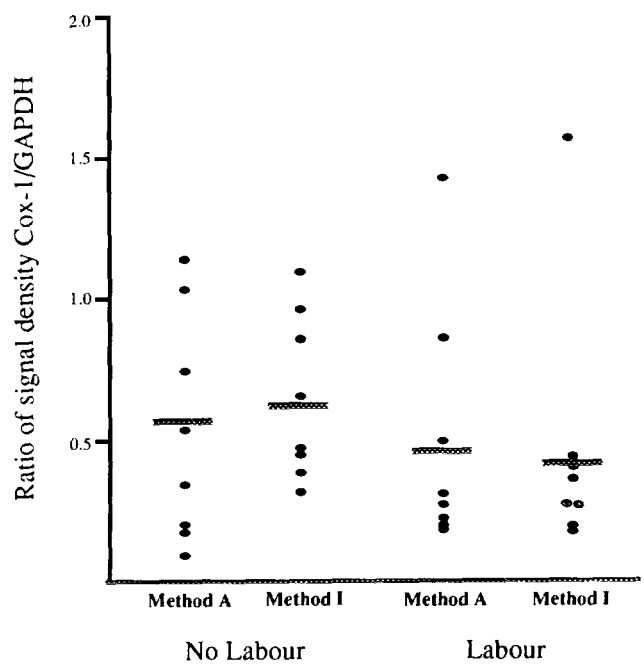

Figure 1.
Differential RNA extraction. Expression of COX-1 in human amnion from pre and post labour deliveries using a differential mRNA extraction methodology. Mean and individual ratio of signal density values (COX-1/GAPDH) from pre and post labour groups. n=8.

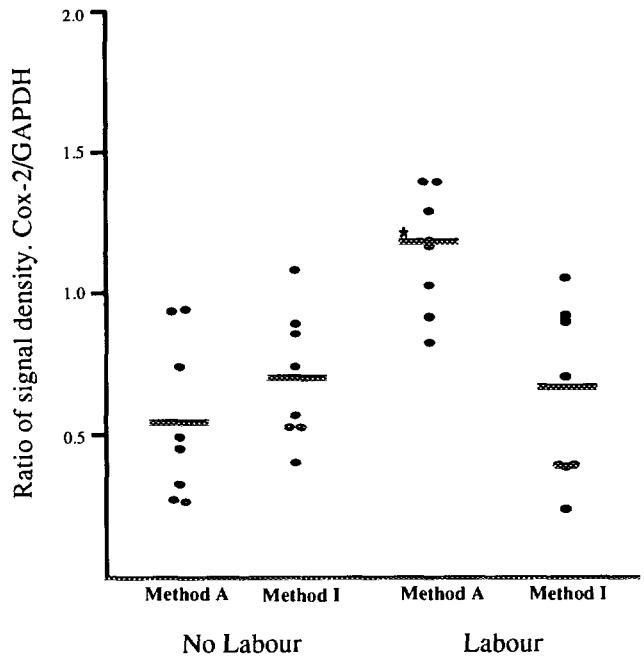

Figure 2.
Differential RNA extraction. Expression of COX-2 in human amnion from pre and post labour deliveries using a differential mRNA extraction methodology. Mean and individual ratio of signal density values (COX-2/GAPDH) from pre and post labour groups. n=8. *p<0.005.

mRNA increases in amnion in association with the onset of labour (4,10). This is consistent with a general increase in the production of prostaglandins that is reported at term labour (1,2). We have previously shown that the expression of COX-2 mRNA is approximately 100 fold higher in amnion at term than that of the COX-1 mRNA (14), and also that the COX-2 mRNA expression increases in association with the onset of labour (10). This data confirms our previous findings.

We had originally postulated that before labour a protein may bind to COX mRNA preventing translation, but following the onset of labour this protein disassociates to allow increased synthesis of COX-2 mRNA. In order to test this theory of 'protein binding' to inhibit COX-2 mRNA expression the differential mRNA extraction methods described by Bailey and Verma 1991 were employed. The two procedures result in either the extraction of 'free' mRNA only (ie mRNA not associated with a protein) or of 'free + bound mRNA' (ie all mRNA within the cell). Before labour there was no difference in the relative levels of expression of COX-2 between the two methods, ie the amount of 'free' mRNA was equal to the amount of 'free + bound' mRNA. However, after labour an overall increase in COX-2 mRNA was found, use of these differential RNA isolation techniques enabled us to show that the increased level of COX-2 mRNA was due to message apparantly bound with protein rather than simply an increase in the free (unbound) mRNA. This is in marked contrast to the mechanism of dexamethasone induced down-regulation of COX seen in vascular smooth muscle cells (9).

Thus, contrary to our original hypothesis, the overall increase of COX-2 message levels seen with labour seems to be in conjunction with an associated protein fraction. This potential COX-2

binding protein (C2BP) could be involved in increased mRNA stability, enhancement of translation initiation or translation elongation.

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