Vol. 198, No. 1, 1994
January 14, 1994
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 304-308

The relative abundance of type 1 to type 2 cyclo-oxygenase mRNA in human amnion at term

Donna Slater*, Louise Berger, Robert Newton, Gudrun Moore, and Phillip Bennett

Action Research Laboratory for the Molecular Biology of Fetal Development, Royal Postgraduate Medical School, Institute of Obstetrics and Gynaecology, Queen Charlottes and Chelsea Hospital, Goldhawk Road, London W6 OXG, UK

Received November 29, 1993

The onset of labor in humans is associated with an increase in prostaglandin production. One of the key steps is the conversion of arachidonic acid to prostaglandin E_2 by cyclo-oxygenase (Cox). Cox has been found to exist as two distinct genes, Cox-1 and Cox-2. We have used RT-PCR to study the relative abundance of mRNA from each Cox gene in amnion at term. Quantitation of PCR efficiency indicated an approximate 100 fold excess of Cox-2 messenger RNA over that for Cox-1. These data point to the importance of Cox-2 in the increased prostaglandin synthesis associated with labor. Further studies should therefore focus on the control mechanisms for Cox-2.

The fetal membranes are thought to play a central role in the synthesis of prostaglandins in association with human parturition. Prostaglandin synthesis has been shown to increase at term in association with labor (1, 2). Initially, precursor arachidonic acid is liberated from intracellular storage by the action of phospholipases (3). Free arachidonic acid then becomes a substrate for cyclo-oxygenase and is converted into prostaglandin H_2. Overall substrate turn-over at the cyclo-oxygenase step is probably the principle factor controlling prostaglandin synthesis in amnion at term (4). The short half life of cyclo-oxygenase (5) suggests a need for continual resynthesis of the enzyme.

Two cyclo-oxygenase enzymes have been described (6, 7). The first, type 1 cyclo-oxygenase (Cox-1), is thought to be constitutively expressed (8). The second, type 2 cyclo-oxygenase (Cox-2), has been more recently described (7) and *in vitro* analysis has shown that its expression in human amnion cells is induced by interleukin-1ß (9).

Traditionally, Northern analysis has been used to study the expression of genes. In this instance, because of the low abundance of cyclo-oxygenase transcripts, we used reverse transcriptase polymerase chain reaction (RT-PCR) (10). RNA is reverse transcribed to produce single stranded cDNA copies and PCR is carried out using gene specific primers. The number of PCR cycles needed to first visualise the amplification product is dependent on the initial concentration of the cDNA synthesised from the starting RNA.

* To whom correspondence should be addressed.

0006-291X/94 $5.00

RT-PCR, whilst in essence a qualitative technique can, with the appropriate controls, be used in a semi-quantitative manner. We have analysed both Cox-1 and Cox-2 expression, using RT-PCR on RNA from amnion cells at term, both before and after spontaneous delivery.

MATERIALS AND METHODS

Human fetal amnion tissue was obtained from term pregnancies and total RNA isolated (11). RNA samples (1μg) were denatured at 70°C for 5 minutes before cooling to 37°C. Random hexanucleotide primers ((0.2μg), 1x reverse transcriptase buffer (BRL), 10 mM dithiothreitol, 1unit RNAse inhibitor (Promega), 1 mM each dNTP and 40 units of M-MLV reverse transcriptase (BRL) were added to a final volume of 20 μl and incubated at 37°C for 60 minutes. The reverse transcription reaction was stopped by heating at 95°C for 5 minutes.

PCR amplification, from the reverse transcribed cDNA,was performed using primers specific for either Cox-1 (12) or Cox-2 (7). PCR was performed using a 1/20 volume of the RT reaction, 1.5 mM magnesium chloride, 0.2mM deoxynucleotide triphosphates (dNTPs), 0.25μg of each primer, and 1 unit of Biotaq polymerase (Biotaq) in a final volume of 50μl. Reaction cycles were; denaturing at 94°C for 1 minute, annealing at 58°C for 1.2 minutes and extension at 72°C for 1 minute for the appropriate number of cycles followed by a 5 minute extension time at 72°C. Aliquots of the PCR reactions (10μl) were separated on 1.2% agarose gels and used for Southern analysis according to standard procedures (13). Filters were hybridised with cloned cDNAs for either human Cox-1 and Cox-2, as appropriate, to confirm the identity of the products.

A PCR reaction, for both Cox-1 and Cox-2, was used to clone the product into Bluescribe (Stragtagene, La Jolla, CA) and the complete DNA sequences were determined. These were found to correspond completely to the published sequences.

To test the PCR sensitivity for amplification of Cox-1 and Cox-2, plasmid DNA was prepared from both Cox-1 and Cox-2 PCR clones. The yield was determined spectrophotometrically, and visualised by agarose gel electrophoresis. Step-wise (1 in 10) dilution series' were prepared for each plasmid to give equivalent concentrations of each target sequence and PCR performed at 25 and 32 cycles, for Cox-2 and Cox-1 respectively (as above).

RESULTS

Initially, it was necessary to establish the number of PCR cycles at which a product for each gene first becomes visible. Typically, a DNA band consisting of around 10ng of DNA will just become detectable by eye on ethidium stained gels. The reverse transcription reaction was carried out on a representative amnion RNA sample and PCR for both Cox-1 and Cox-2 performed for varying numbers of cycles (Figure 1). An amplification product for Cox-1 first appeared at 32 cycles, whilst for Cox-2 only 24-26 cycles were required. To confirm this result, we performed RT-PCR on a further 20 amnion RNA samples collected at term (data not shown). For Cox-1, after 32 cycles amplification, a product was just detectable in 13 samples. In a further 7, the product level was still below the detection limit of the ethidium stained gel. After 25 cycles amplification, for Cox-2, products were just detectable in 12 samples and 8 remained below the limit of gel sensitivity. Southern hydridisation, with Cox-1 or Cox-2 as appropriate, confirmed the presence of amplification products in all samples. Thus in most cases amplification products were just visible after 32 PCR cycles for Cox-1 and 25 cycles for Cox-2. At this point amplification is occurring exponentially and the final product concentration is proportional to the starting target cDNA concentration. If each gene is amplified equally, this difference in cycle number implies that Cox-2 message is around 128 (2^7) times more abundant than Cox-1. In reality the efficiency of the PCR will most likely be less than the theoretical maximum of doubling each cycle. Additionally, amplification of each target gene may occur at different rates depending on its sequence.

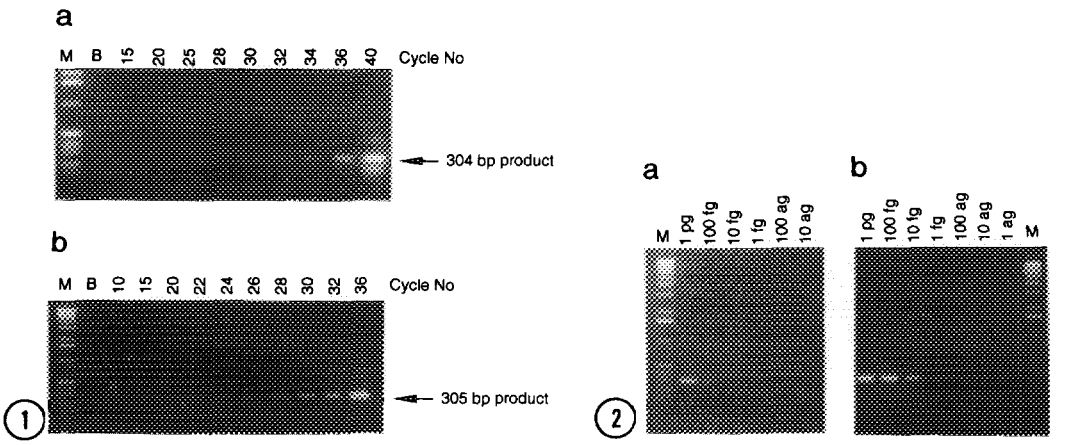

Figure 1.

PCR cycle profiles for a) Cox-1 and b) Cox-2. PCR reactions (for Cox-1 and Cox-2 respectively) were set up and carried out for the number of cycles indicated. Aliquots (10µl) of reaction products were run on 1.2% agarose gels and ethidium bromide stained to allow visualisation of amplification products (as described in text). Gel lanes are; M = 1 kb ladder, B = blank (PCR with no template as negative control), then increasing numbers of PCR cycles as indicated.

Figure 2.

Target DNA concentration gradients for a) Cox-2 at 25 cycles and b) Cox-1 at 32 cycles of PCR. Dilution series using linearised target DNA for Cox-1 or Cox-2 were set up and PCR carried out on samples as indicated. Aliquots (10µl) of reaction products were run on 1.2% agarose gels and ethidium bromide stained to allow visualisation of amplification products (as described in text). Gel lanes are; M = 1 kb ladder and increasing quantities of target as indicated (1 pg =1 x 10^{-12}g, 1 fg = 1 x 10^{-15}g, 1 ag = 1 x 10^{-18}g).

In each case, it was therefore necessary to make an evaluation of the PCR sensitivity for each of Cox-1 and Cox-2 under the conditions used. From the Cox-2 plasmid dilution series, PCR was performed using 25 cycles (Figure 2a). An amplification product is clearly visible using only 10 fg of Cox-2 target DNA. PCR was also performed at 32 cycles for the Cox-1 plasmid dilution series (Figure 2b). With only 1 fg of Cox-1 target DNA, an amplification product was easily observed. However, at 0.1 fg of target sequence Cox-1 yielded a barely discernible PCR product.

DISCUSSION

Initially, we found that PCR amplification, from a single amnion sample collected following labour, resulted in visible amplification products first occurring after 32 cycles for Cox-1 and 25 cycles for Cox-2. The limit of resolution on agarose gels (in our hands) is around 10ng of DNA per band. The implication of this result is that Cox-1 message is present at considerably lower levels than that for Cox-2. We repeated the test on a further 20 samples at term. On average RT-PCR resulted in visualisation of Cox-1 products at around 32 cycles, whilst typically for Cox-2 only 25 cycles were required. Assuming a doubling with each PCR cycle and equal amplification efficiency, this result indicates a 128 fold higher concentration of Cox-2 message over Cox-1.

To validate this finding, an assessment was made of the relative sensitivities of each amplification process by amplification from cloned products of known target DNA

concentrations. It was found that using 25 PCR cycles for Cox-2, we could just detect a product on agarose gels with as little as 10 fg of target DNA. With 32 cycles PCR, we were able to observe a band on agarose gels using as little as 0.1 fg starting target for Cox-1. Assuming similar sensitivities for the RT-PCR experiment, these data imply a relative abundance of Cox-2 to Cox-1 of the order of 100:1.

The above quantitation relates to PCR from a double stranded plasmid DNA template. In the RT-PCR experiments, we were performing PCR from a single stranded hexanucleotide primed cDNA. This may be less efficient than PCR from a double stranded target. A further problem is that reverse transcription from mRNA to cDNA may not be 100% efficient. Because subsequent PCR can only detect cDNA, the actual level of each mRNA species may therefore be higher than that estimated for the cDNA. Since aliquots of the same RT reaction were used for both the Cox-1 and Cox-2 PCR, comparison of the cDNA levels is likely to be a true reflection of the relative levels of mRNA. The quantitation, at 32 and 25 cycles respectively, also gives a good idea of the sensitivities of Cox-1 and Cox-2 PCR. This provides information on the order of magnitude of the absolute levels of each mRNA as well as an estimate of their relative abundance.

These data, whilst only giving order of magnitude figures, clearly show that Cox-2 is expressed at a considerably higher level than Cox-1 in human amnion at term. It is therefore the type 2 enzyme which is more likely to play the greatest role in the synthesis of prostaglandins in this tissue with labor. A possible model is that, in an unstimulated state, the constitutively expressed Cox-1 gene is responsible for low level conversion of arachidonic acid to prostaglandins seen in amnion prior to labour. On stimulation, for example towards the end of pregnancy at parturition, expression of the inducible Cox-2 gene may be up-regulated to allow greater synthesis of prostaglandins. In this paper we have described evidence showing that at term, the level of Cox-2 expression is considerably higher than the level for Cox-1.

Many of the factors which stimulate prostaglandin synthesis in amnion are not yet understood. Our findings indicate the importance of Cox-2 and undoubtedly a fuller understanding of the factors which control the expression of Cox-2 will provide valuable information about the mechanisms of parturition.

ACKNOWLEDGMENTS

This work was supported by grants from Birthright (B1/91) and the MRC.

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