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Nitric Oxide Synthase mRNA Expression in Human Fetal Membranes: A Possible Role in Parturition

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Nitric oxide (NO) is a potent endogenous smooth-muscle relaxant. It is synthesised from l-arginine by isoforms of nitric oxide synthase (NOS). Whilst it is clear that the uterus responds to NO by relaxation, NOS expression has not been investigated in fetal membranes or myometrium in human pregnancy. This study has shown, using semi-quantitative RT-PCR, expression of cNOS mRNA in human amnion, chorion-decidua, and placenta. iNOS mRNA expression was demonstrated in human amnion, chorion-decidua, and placenta. It is possible that NO synthesised in fetal membranes may act either directly to inhibit myometrial contractility or indirectly to interact with other labour-associated genes, such as cyclo-oxygenase, to coordinate the onset of labour.

The mechanisms that initiate the onset of labour in humans are poorly understood. It is our hypothesis that parturition occurs through the up-regulation of a group of pro-labour genes, such as oxytocin receptors and gap junction proteins. We have previously shown (1) that labour is associated with increased expression of cyclo-oxygenase (COX), the central enzyme in prostaglandin synthesis. With labour there is increased expression of the inducible, type-2 isoform, with no change in expression of the constituitive, type-1 isoform.

NO is a highly reactive inorganic free radical with widespread biological effects. It inhibits platelet aggregation, is a potent smooth muscle relaxant, and can act as a cellular messenger. Its effects on smooth muscle are mediated via stimulation of the cyclic guanylate cyclase pathway. Both animal and human studies (2,3) have shown that there is a NO-cGMP relaxation pathway in the uterus with decreased responsiveness to NO at term, suggesting that NO may decrease uterine contractility, maintaining the uterus in a state of quiescence during pregnancy.

NO is synthesised by oxidative de-amination of the semi-essential amino acid L-arginine by isoforms of the transcriptionally regulated enzyme nitric oxide synthase (NOS,EC1.14.23) (4). NOS exists in at least three isoforms: a constituitive, calcium-dependent endothelial form (cNOS), a constituitive, calcium-dependent neuronal form (nNOS) and an inducible, calcium-independent form (iNOS). NOS activity in myometrium has been demonstrated in animal studies, and been shown to fall both on the last day of pregnancy (5) and with the onset of labour (6). Telfer et al has identified cNOS in non-pregnant human endometrium and myometrium.

Whilst it is clear that NO relaxes myometrial smooth muscle, the production of NO by either fetal membranes or myometrium itself has not been directly studied in human pregnancy. This study investigates the expression of cNOS and iNOS in term fetal membranes.

MATERIALS AND METHODS

Human term fetal membranes and placenta were collected (n=12), washed with phosphate buffered saline, split into amnion, chorion-decidua, and placental trophoblast, and immediately snap frozen in liquid nitrogen. Human umbilical endothelial cells (HUVEC) a positive control for cNOS, were obtained from established culture. RNA was extracted using a standard guanidium isothiocyanate technique (7). RNA samples (1μg) were denatured at 70°c for 5 minutes and cooled to 37°c. Reverse transcription was carried out using random hexanucleotide primers (0.2μg), ×1 reverse transcriptase buffer, 10mM dithiothreitol, 1mM each dNTP, 40units M-MLV reverse transcriptase (Gibco BRL) and 1unit RNAse inhibitor (Pharmacia Biotech) were added to a final volume of 20μl and incubated at 37°c for 60 minutes. Reverse transcription was terminated by heating to 90°c for 5 minutes.

PCR amplification from reverse transcribed cDNA was carried out using specifically designed PCR primers for cNOS and iNOS: cNOS sense: 5′-GCACAGGAAATGTTCACCTAC-3′, cNOS antisense: 5′-CACGATGGTGACTTTGGCTAG-3′ (8), iNOS sense: 5′-GAGCTTCTACCTCAAGCTATC-3′, iNOS antisense: 5′-CCTGATGTTGCCATTGTTGGT-3′ (9). PCR amplification for Glyceraldehyde-3-mono-

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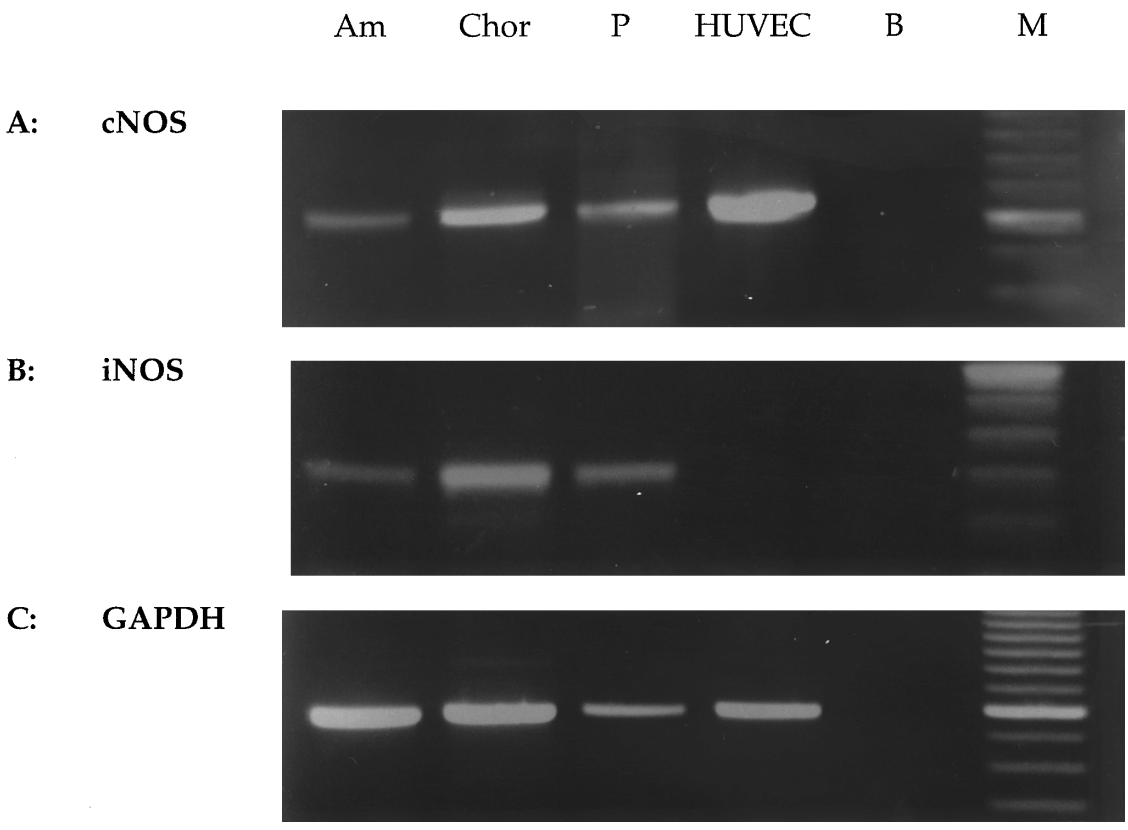

FIG. 1. Expression of NOS isoforms and GAPDH (as control) in term human amnion (Am), chorion-decidua (Chor), placenta (P), and human umbilical vein endothelial cell (HUVEC). B, Blank (PCR with no DNA template); M, 100bp DNA ladder.

phosphate dehydrogenase (GAPDH) was carried out on the same samples as a parallel control. GAPDH sense: 5′-CCACCCATGGCA-AATTCCATGGCA-3′, GAPDH antisense: 5′-TCAAGACGGCAG-GTCAGGTCCACC-3′ (10).

PCR was performed on a 1/40 volume of the RT reaction with 1.5mM magnesium chloride, 0.2mM dNTPs, 125ng of each primer, and 1unit of Biotaq polymerase (Biotaq) in a final volume of 25μl. Reaction cycles were an initial denaturation step of 4 minutes at 94°c, followed by denaturation at 94°c for 30 seconds, annealing at 55°c (cNOS) or 62°c (iNOS) for 30 seconds, and extension at 72°c for 30 seconds for the appropriate number of cycles, followed by a further 5 minute extension time at 72°c. 10μl aliquots of the PCR product were separated by horizontal gel electrophoresis, on a 1% agarose ethidium bromide stained gel. DNA was transferred to a nylon transfer membrane (MSI) for Southern blotting. Filters were hybridised with [^{32}P]dCTP labelled cDNA probes, for cNOS, iNOS and GAPDH and washed to high stringency. Autoradiography was carried out to confirm the identity of the PCR products. PCR products were initially sub-cloned, ligated into pGEM-T vector (Promega) and subsequently sequence verified.

For each target gene, cycle profiles were carried out on pooled samples to determine the exponential phase of amplification, where final product concentration is proportional to starting cDNA concentration. Cycles were carried out from 25 to 60, at 5 cycle intervals. Since the initial amount of RNA template in each case is identical (1μg), semi-quantitation is achieved.

RESULTS

cNOS expression was shown in amnion, chorion-decidua, placenta and HUVEC (positive control). The expected product size was 654bp. Figure 1A. shows cNOS PCR amplification at 55 cycles. Visually there was increased amplification product concentration from chorion-decidua. This was confirmed by cycle profiles which indicated an optimal cycle number for amnion as 52, and for chorion-decidua as 45 (data not shown).

iNOS expression was shown in amnion, chorion-decidua and placenta. Expected product size was 313bp. Figure 1B. shows iNOS amplification at 45 cycles. There was no iNOS expression in HUVEC cells. In a similar expression pattern to cNOS there appeared to be increased amplification product concentration from chorion-decidua and this was confirmed by cycle profiles, indicating optimal cycles for amnion as 45 and for chorion-decidua 40 (data not shown).

GAPDH PCR amplification (expected product size 598bp) confirmed the presence of cDNA starting template and amplification products (Figure1C).

PCR products were subsequently verified by Southern hybridization to previously generated and sequenced products.

DISCUSSION

This study has for the first time demonstrated the expression of cNOS and iNOS mRNA in human fetal

membranes at term. In addition cycle profiles indicate an approximate 100 fold higher amplification product concentration of cNOS mRNA in chorion-decidua compared to amnion, and higher levels of iNOS expression in all tissues examined with an approximate 30 fold higher concentration of iNOS mRNA in chorion-decidua compared to amnion.

The role of NO generated from NOS expression within fetal membranes is speculative. NO might exert a direct effect on myometrial contractility, although the short half-life of NO (4 seconds) and anatomical considerations might suggest this unlikely. We propose that NO might interact with other labour-associated genes exerting an indirect effect on myometrial activity and possibly the initiation of labour. Prostaglandins have been shown to play a pivotal role in the onset of parturition. Prostaglandins are generated from arachidonic acid by the actions of cyclo-oxygenase. At present two cyclo-oxygenase enzymes have been identified, a constituitive isoform COX-1 and an inducible isoform COX-2. We have shown previously (1) that fetal membranes, and amnion in particular, express COX-2 and are a significant source of PGE_2. Swierkosz et al. (11) have shown in endothelial cells that low levels of NO induce COX whilst high levels inhibit. In addition Tetsuka et al. (12) have proposed that in rat mesangial cells iNOS-generated NO activates cyclo-oxygenase, resulting in the production of PGE_2 which, via the EP_1 receptor, then down-regulates iNOS. It is possible that a similar pathway is active within these tissues and that changes in NOS expression and therefore NO levels may influence PGE_2 production and the onset of labour.

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