Molecular Human Reproduction Vol.7, No.6 pp. 581–586, 2001

Human labour is associated with nuclear factor-κB activity which mediates cyclo-oxygenase-2 expression and is involved with the 'functional progesterone withdrawal'

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Human labour is associated with the up-regulation of prostaglandins within the uterus, synthesized via the type-2 cyclo-oxygenase enzyme (COX-2). These lead to remodelling of the fetal membranes and cervix and to stimulation of myometrial contractions. In the human, the principal source of prostaglandins is the amnion. Progesterone acts to promote myometrial quiescence, and in many species the onset of labour is preceded by withdrawal of progesterone. Humans show no systemic progesterone withdrawal, although biochemical changes within the uterus are similar to those in other species. A mutual negative interaction between the transcription factor nuclear factor (NF)-κB and the progesterone receptor (PR) has been reported. Using transient transfections and assays for transcriptional activation and promoter binding, we have shown that there is constitutive activity of NF-κB in amnion cells at the time of labour, and that COX-2 expression depends upon NF-κB. In cells obtained before labour, in which NF-κB activity is low, increasing the concentration of PR represses NF-κB dependent transcription, while stimulation with IL-1β both increases NF-κB activity and represses PR activity. Our data suggest that human labour is associated with constitutive NF-κB activity within the amnion, which functions to increase the expression of COX-2 and appears to contribute to the 'functional progesterone withdrawal'.

Key words: COX-2/labour/NF-κB/parturition/progesterone

Introduction

Prior to labour, the cervix remodels to allow effacement and dilatation, and the uterus begins to contract. The biochemical events involved in this process include increased levels of interleukin-1β (IL-1β) (Romero *et al*., 1990) and prostaglandin synthesis (Mitchell *et al*., 1995). The amnion is the principal site of prostaglandin synthesis in the human and the central enzyme involved in their synthesis is cyclooxygenase (COX) which converts free arachidonic acid into the prostaglandin precursors PGH_2 and PGG_2. Two isoforms of the COX enzyme have been described, COX-1 and COX-2. COX-2 is rapidly inducible and it is this isoform that has been shown to increase in association with human labour (Slater *et al*., 1999).

For more than 60 years the role of the 'pro-pregnancy' hormone, progesterone, as an inhibitor of uterine contraction and cervical ripening has been recognized. In 1965 Csapo referred to the 'progesterone block' which opposes the excitatory effect of oestrogen and prevents myometrial responsiveness to uterotonic agents and allows pregnancy maintenance (Csapo and Pinto-Dantas, 1965). For labour to occur, however, this 'progesterone block' must be overcome. In many species the onset of labour is preceeded by the withdrawal of progesterone. However, in humans there is no detectable progesterone withdrawal, although there is up-regulation of a range of pro-labour genes which are normally repressed by the presence of progesterone (Chwalisz *et al*., 1994; Lye, 1994). It has been suggested that these events may be mediated by changes in the function of the progesterone receptor (PR) rather than by withdrawal of progesterone itself (Mitchell and Wong, 1993; Chaim and Mazor, 1998). The term 'functional progesterone withdrawal' has therefore been used to describe the indirect withdrawal of progesterone through an effect of the receptor.

The transcription factor nuclear factor κB (NF-κB) has been implicated in the regulation of pro-labour genes including inflammatory cytokines and COX-2 (Dokter *et al*., 1995; Newton *et al*., 1997; Wu *et al*., 1997). NF-κB is a transcription factor whose effect on transcription is known to be regulated by cytokines such as IL-1β (Croston *et al*., 1995).

NF-κB functions as homo- or heterodimers of the Rel family of proteins, which includes p50, p65, c-Rel, p52 and RelB.

The most common combination of subunits is a heterodimer of the p50 and p65 proteins. NF-κB normally exists in the cytoplasm of cells bound by a member of the inhibitor kappa B (IκB) protein family. NF-κB activation by inducers, such as IL-1β, leads to phosphorylation and degradation of the IκB protein allowing NF-κB translocation to the nucleus where it can then bind to specific sites within the promoter sequence of target genes (Baldwin, 1996).

A mutual negative interaction in which NF-κB represses PR function and vice versa has been described (Kalkhoven *et al*., 1996). We postulated that NF-κB may play a role in the biochemical events associated with labour acting both to up-regulate 'inflammatory' mediators, in particular COX-2, and to suppress PR function and thereby contribute to progesterone withdrawal. We have therefore studied basal and IL-1β stimulated NF-κB activity in amnion cells in association with labour. We have also explored the role of NF-κB in regulating COX-2 expression and examined the possibility of interaction with PR.

Materials and methods

Cell preparation

Tissues were obtained from patients at term either at elective Caesarean section prior to labour, or following spontaneous labour onset and vaginal delivery. Ethics committee approval and patient consents were obtained prior to the study. Amnion cells were prepared from tissue as previously described (Bennett *et al*., 1987). In brief, amnion was separated from the chorion and washed in phosphate-buffered saline (PBS). The membrane was cut into strips and incubated in 0.5 mmol/l EDTA (BDH) for 15 min at 20°C. The strips were washed in PBS and then incubated in Dispase (Life Technologies, Paisley, UK) 2.5 g/l for 40 min, 37°C. Amnion epithelial cells were separated by vigorous shaking for 3 min and strips removed. Cell pellets were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Sigma, Poole, UK), 2 mmol/l L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies).

Plasmids

The NF-κB-dependent reporter, pGL3.6κB.BG.luc, contains six copies of the decameric NF-κB binding site upstream of a minimal β-globin promoter driving a luciferase reporter. Control constructs were: pGL3.BG.luc containing only the minimal β-globin promoter and pGL3.6κBmut.BG.luc containing six mutated copies of the NF-κB binding site (Nasuhara *et al*., 1999). The COX-2 promoter construct, pGL3.C2.2.luc, was created by cloning the promoter fragment (-2375/+43) into pGL3 basic vector (Promega, Southampton, UK) and was then used to create the mutated constructs, pGL3.C2.2.NF1.mut (mutated at the downstream NF-κB binding site) and pGL3.C2.2.NF2.mut (mutated at the upstream NF-κB binding site). The progesterone-dependent reporter, pGL3.3PRE.luc, contains three copies of the progesterone response element driving a luciferase reporter gene. hPR0 is an expression vector for the PR B isoform (Kastner *et al*., 1990).

Nuclear protein extracts

Extracts were prepared according to a published method (Dignam *et al*., 1983). In brief, amnion cells were washed in PBS before being resuspended in buffer A on ice for 10 min, then centrifuged for 10 min at 14 000 *g*, 4°C. The pellet was resuspended in buffer C and incubated on ice for 1 h, with agitation before centrifugation for 10 min at 14 000 *g*, 4°C. Nuclear proteins were resuspended in 4 volumes of buffer D and stored at –80°C.

Electrophoretic mobility shift assays (EMSA)

Nuclear protein 10–20 μg, were used in binding reactions as described previously (Dignam *et al*., 1983) with the consensus NF-κB binding site as the probe (Promega). Specificity was determined by competition with 100-fold excess of non-radiolabelled probe. Supershift analysis was performed by the addition of antisera to p65, p50, p52, RelB and cRel (Santa Cruz, CA, USA), on ice, 90 min prior to addition of labelled probe. Electrophoresis was performed on a 6% non-denaturing acrylamide gel in 0.25×Tris-borate-EDTA (TBE). Gels were dried and protein–DNA complexes were visualized by autoradiography.

Transient transfections

Transfection was achieved using the lipid carrier Tfx-50 (Promega) which was prepared according to manufacturer's instructions. 2.5×10^5 cells were seeded into 24-well plates and allowed to grow to 85–90% confluence. Transfections used a charge ratio of 3:1, 1.0 μg DNA/well and 2 h incubation time. In co-transfection studies, 0.5 μg of each vector was used. Cells were cultured for a total of 48 h, treated in the final 8 h of this period, then lysed using passive lysis buffer (Promega) and stored at –80°C until analysis using Luciferase assay buffer (Promega). All samples and assay buffers were allowed to reach room temperature before analysis. Samples were centrifuged to 30 s at 200 *g*. Samples and buffers were mixed gently and the light released was measured by a luminometer (Turner design TD 20/20; Promega). Consistent transfection efficiencies were indicated, as luminometer readings in each experiment were very similar.

Site-directed mutagenesis

This was performed using the Gene Editor kit (Promega) following the manufacturer's instructions. An oligonucleotide containing the relevant mutation was phosphorylated and annealed to alkali-denatured vector. T4 DNA polymerase and T4 DNA ligase were added and incubated for 90 min, 37°C to allow synthesis of the second strand. BMH 71-18 mutS competent cells were transformed with 10 ng of vector and cultured overnight. DNA was prepared and used to transform JM109 cells. Sequence analysis verified the mutation. Oligonucleotides used were NF1mut 5′-CAGGAGAGTGGCCACTACCCCC-3′ and NF2mut 5′-CGGGAGAGGCCATTCCCTGC-3′. The bases chosen for mutation have been previously demonstrated to be critical for protein–DNA binding (Newton *et al*., 1997).

Statistical analysis

Statistical analysis was by analysis of variance with post-hoc testing using Fisher's protected least significant difference test.

Results

To determine transcriptional activity due to NF-κB in association with labour, amnion cells were transiently transfected with the NF-κB-dependent reporter construct, pGL3.6κB.BG.luc. In pre-labour amnion cells, reporter activity was significantly increased in IL-1β-treated cells compared to unstimulated cells ($P = 0.003$, Figure 1). In post-labour cells, unstimulated NF-κB-driven reporter activity is significantly higher than in pre-labour cells ($P = 0.0001$), but was not further increased by IL-1β treatment (Figure 1). EMSA studies using nuclear proteins extracted from amnion cells demonstrated the ability of proteins present in the nucleus to bind to an oligonucleotide of the consensus NF-κB DNA binding site. In nuclei from

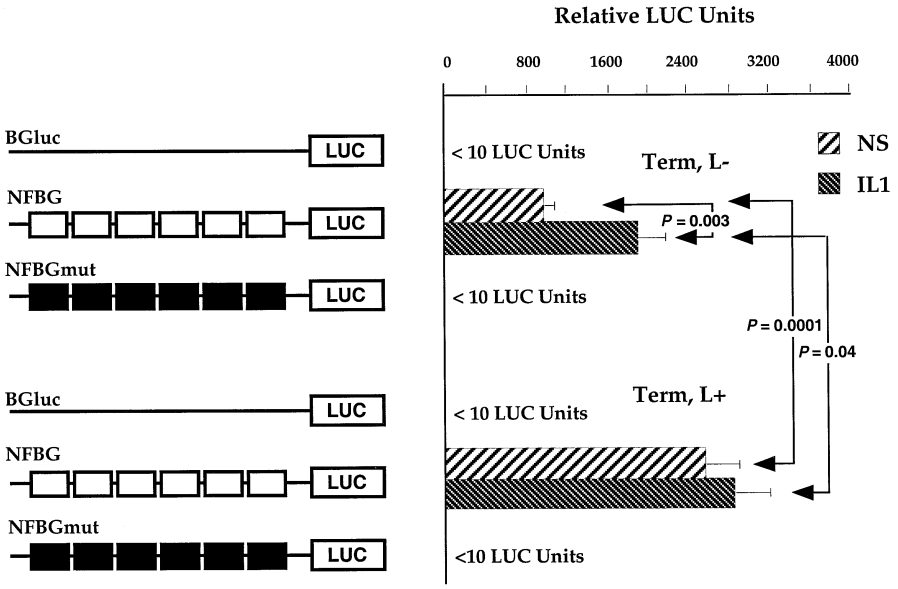

Figure 1. Labour and interleukin (IL)-1β-induced nuclear factor (NF)-κB-dependent transcription. Pre- and post-labour cells transiently transfected with pGL3.6κB.BG.luc were either not stimulated (NS) or stimulated with IL-1β (1 ng/ml) 8 h prior to luciferase assay. Data (n = 4) from assays each performed in triplicate are expressed relative to untreated control construct and plotted as means ± SE. Significant increases in reporter expression compared to non-stimulated or IL-1β-treated are indicated. No significant luciferase activity was seen following transfection of either empty vector containing no NF-κB binding sites or vector containing six mutated NF-κB binding sites.

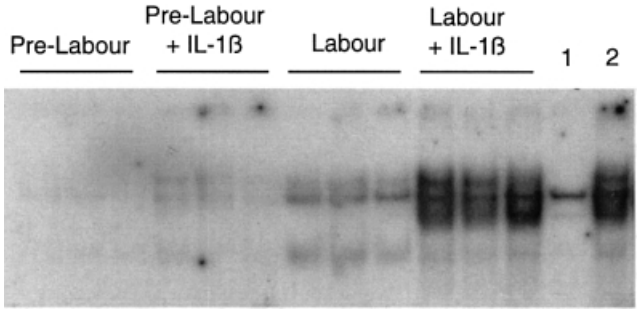

Figure 2. Labour and interleukin (IL)-1β induced nuclear factor (NF)-κB DNA binding. Electrophoretic mobility shift assay was performed using nuclear extracts from pre- or post-labour cells treated for 30 min with no stimulant (NS) or IL-1β (1 ng/ml). Three band shifts due to protein binding are shown. Specific NF-κB binding is shown by competition with 100-fold excess non-radioactive consensus NF-κB oligonucleotide (lane 1) compared to 100-fold excess of non-radioactive OCT-1 oligonucleotide (lane 2).

pre-labour cells there was very little protein binding but this was increased by IL-1β stimulation. In extracts from post-labour amnion, protein binding in unstimulated cells was significantly greater than in pre-labour cells. DNA binding was further increased in nuclei from post-labour cells by IL-1β treatment (Figure 2). Specificity was shown by competition with a 100-fold excess of non-radioactive consensus NF-κB oligonucleotide (lane 1, Figure 2) compared to a 100-fold excess of non-radioactive OCT-1 oligonucleotide (lane 2, Figure 2). Three authentic band shifts were seen on EMSA analysis using NF-κB consensus oligonucleotides, and have been designated as 'a', 'b' and 'c'. Band 'a' was supershifted only by antibodies to p65. Bands 'b' and 'c' were supershifted by antibodies to p65 and p50 (Figure 3).

The possibility that NF-κB may be involved in the regulation of the COX-2 promoter was studied using the constructs of

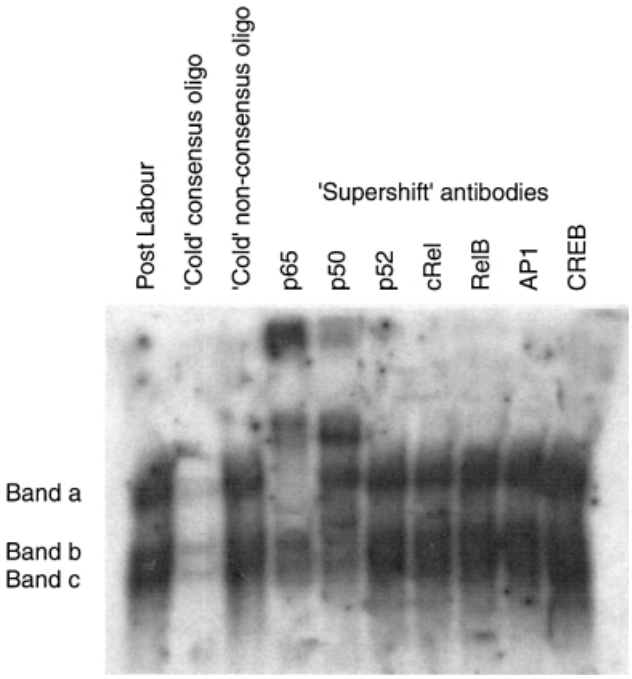

Figure 3. Nuclear factor (NF)-κB subunits involved in DNA binding. To determine which NF-κB subunits are involved in DNA binding, electrophoretic mobility shift assay was performed using nuclear extracts from post-labour amnion cells incubated with antibodies to p65, p50, p52, RelB or cRel. Specificity was shown by competition with a 100-fold excess non-radioactive consensus NF-κB oligonucleotide (lane 2) compared to 100-fold excess of non-radioactive OCT-1 oligonucleotide (lane 3). Supershifts only occurred with antibodies to p65 and p50. Antibodies to AP-1 and CREB demonstrated the specificity of the supershift.

the COX-2 promoter, containing wild-type and mutated NF-κB sites, transfected into post-labour amnion cells. Mutation of the downstream NF-κB DNA binding site (NF1mut) significantly

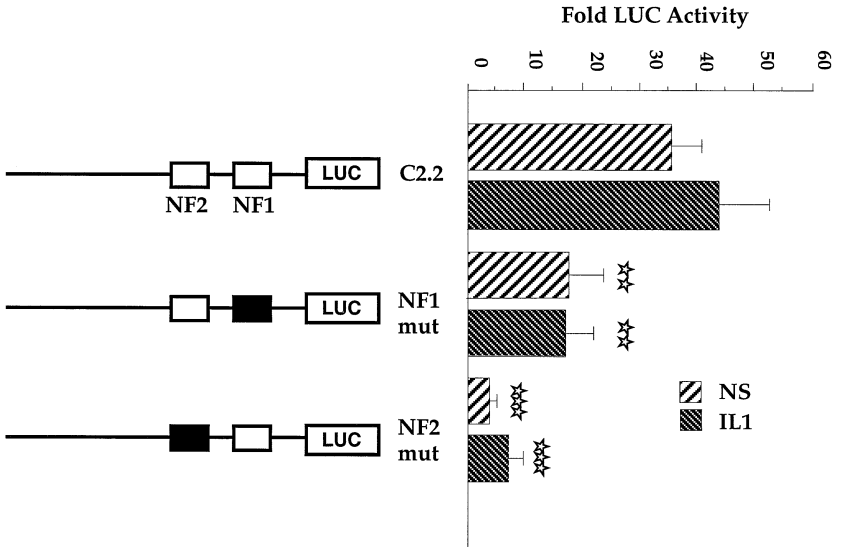

Figure 4. Nuclear factor (NF)-κB is required for transcription of the *COX-2* gene. Amnion cells collected post labour were transiently transfected with the wild-type *COX-2* promoter, pGL3.C22.luc (wt), the downstream NF-κB binding site mutant, pGL3.C22.NF1.mut (NF1mut), or the upstream NF-κB binding site mutant, pGL3.C22.NF2.mut (NF2mut). Cells were either not stimulated or stimulated with interleukin (IL)-1β (1 ng/ml) for 8 h prior to cell lysis and analysis. Data ($n = 3$) from assays each performed in triplicate are expressed relative to a control construct containing only a basic promoter and are plotted as means ± SE. Significant repression of transcription compared to the wild-type promoter is represented by two stars ($P < 0.01$) or three stars ($P < 0.0003$).

reduced reporter expression when compared to the wild-type COX-2 promoter. Mutation of the upstream NF-κB DNA binding site (NF2mut) caused a greater reduction (Figure 4) in expression from the COX-2 promoter.

The above data demonstrate that in amnion cells, IL-1β increases the binding of NF-κB to DNA and increases NF-κB-dependent transcription. To study whether the putative repressive effect of NF-κB on PR-dependent transcription occurs in amnion cells, IL-1β treatment of pre-labour cells was used to activate NF-κB. Cells were transiently co-transfected with a progesterone-responsive reporter construct (3PRE.luc) in the presence or absence of excess PR. Progesterone treatment alone caused increased reporter expression with ($P = 0.006$) or without ($P = 0.016$) excess PR (Figure 5A). However, IL-1β, in combination with progesterone, caused a significant repression of reporter expression, to control levels. Excess PR did not negate the repressive effect of IL-1β, although it did increase the response of the reporter to all treatments (Figure 5A). To determine whether this was a mutually repressive effect, cells were transiently co-transfected with the NF-κB-dependent reporter construct, pGL3.6κB.BG.luc, and an expression vector for PR or the control construct. Overexpression of PR caused significant repression of NF-κB-dependent reporter expression ($P = 0.0004$, Figure 5B).

Discussion

This study demonstrates constitutively active NF-κB in amnion cells in association with labour. There is an apparent discrepancy between the increased DNA binding by NF-κB seen in EMSA analysis after IL-1β treatment, and the lack of increase in reporter expression in transfection studies in post-labour cells. This may be because the maximum response from the reporter has been reached. Alternatively, the increase in binding seen in the EMSA may be due to binding of NF-κB that is unable to drive expression of the reporter. It has been demonstrated that degradation of IκB protein cannot fully explain NF-κB activity and that further regulatory steps are involved downstream of DNA binding (Nasuhara *et al*., 1999). Phosphorylation of the p65 subunit of NF-κB is also critical for transcriptional activity, independent of translocation or DNA binding (Egan *et al*., 1999). There are currently ten IκB family members including IκBα, IκBβ1 and IκBβ2. The duration of transcriptional activation by NF-κB is determined by the kinetics of degradation and re-synthesis of the associated IκB protein. IκBα, for example, is involved in transient transcriptional activation as it is rapidly degraded and its synthesis is induced by NF-κB activity. IκBβ degradation, however, is cell and stimuli dependent but the activation so caused is persistent (Thompson *et al*., 1995). IL-1β causes degradation of IκBβ in most cell types, and synthesis of new hypophosphorylated IκBβ then binds to NF-κB and acts as a chaperone by protecting it from association with IκBα. The IκBβ:NF-κB complex can enter the nucleus, bind DNA and activate transcription. However, subsequent phosphorylation of IκBβ or association with high-mobility group protein I (HMG I) converts it from an activator to a repressor of transcription (Tran *et al*., 1997). From our data the IκB involved in regulation of NF-κB in amnion cannot be determined. Involvement of an IκBβ is possible since it binds to the p65 containing complexes which we have identified, but this will require further investigation.

It is clear, however, that basal DNA binding and transcriptional activity due to NF-κB, without IL-1β stimulation, is significantly higher in post-labour amnion cells than in pre-labour cells. Therefore human labour is associated with constitutive NF-κB activity in the amnion. Constitutive activity has been reported previously in carcinoma cell lines and rat

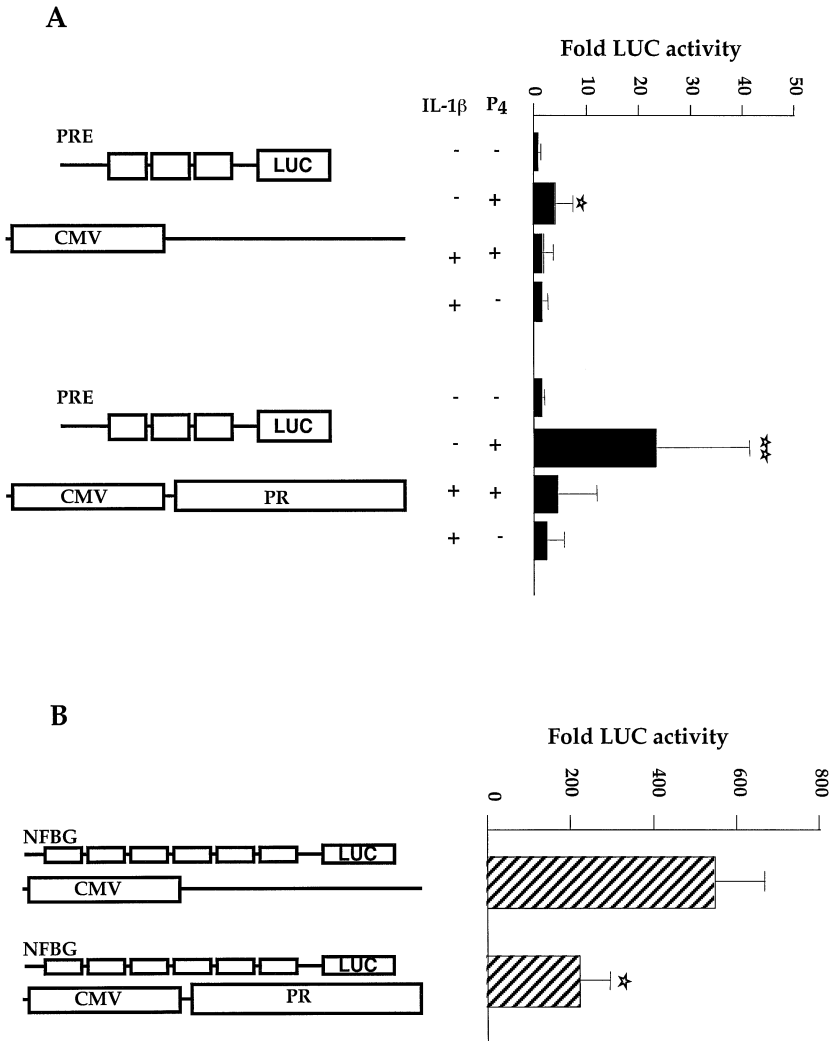

Figure 5. Mutual repression of nuclear factor (NF)-κB and progesterone receptor (PR) exists in amnion cells. (**A**) Pre-labour amnion cells transiently transfected with pGL3.3PRE.BG.luc were co-transfected with either a control or PR expression vector and treated with progesterone (P_4) (10^{-7} mol/l) and/or interleukin (IL)-1β (1 ng/ml). Data ($n = 3$) from assays each performed in duplicate are expressed relative to the control and plotted as means ± SE. Progesterone treatment caused a significant increase in reporter expression compared to untreated cells: $P = 0.006$ (one star) and $P = 0.016$ (two stars). Progesterone and IL-1β treatment in combination with progesterone repressed reporter expression to control levels ($P = 0.045$ and $P = 0.037$ respectively compared to progesterone treatment alone). (**B**) Pre-labour amnion cells transiently transfected with pGL3.6κB.BG.luc were co-transfected with either a control or PR expression vector. Data ($n = 3$) from assays each performed in duplicate are expressed relative to control construct and plotted as means ± SE. Significant repression of transcription was seen in cells with PR expression compared to cells in which PR was not expressed: $P = 0.0004$.

hepatic stellate cells (Bargou *et al*., 1997; Dejardin *et al*., 1999). It was suggested that this could be due to low expression and high turnover of IκBα (Bargou *et al*., 1997). Other explanations may be the chaperone effect of IκBβ, or a similar effect of another IκB family member, which allows DNA binding and activation whilst complexed with NF-κB. Alternatively, the IκB proteins that are available to bind to NF-κB have slow re-synthesis kinetics which allow sustained periods of, albeit transient, NF-κB activity (Elsharkawy *et al*., 1999).

Three NF-κB band shifts were seen on EMSA analysis and we have designated them 'a', 'b' and 'c'. The highest molecular weight band, 'a', is supershifted only by antibodies to p65. This suggests that this band represents binding of homodimers of p65 and that this is the most abundant form of NF-κB present in amnion cells with labour. Antibodies to p65 and p50 caused a supershift of bands 'b' and 'c' suggesting that these consist of heterodimers p65:p50. The IκBβ protein shows a preference for binding to dimers which contain p65. It is possible therefore that the constitutive NF-κB activity seen in amnion during labour is due at least in part to binding of IκBβ protein, although this clearly requires further investigation.

The activation of NF-κB in association with labour onset occurs at a time when COX-2 mRNA and protein levels are known to increase (Slater *et al*., 1995). The COX-2 promoter contains two NF-κB binding sites which have been shown to be important for transcription in several cell types (Newton *et al*., 1997; Wang and Tai, 1998; Belt *et al*., 1999). In A549 cells, there appears to be a preference for binding to the upstream NF-κB site compared to the downstream site (Newton *et al*., 1997). Our data also show that, although both sites play a role in COX-2 expression, the upstream NF-κB site is of greatest importance in amnion cells.

A mutual negative interaction has been described between NF-κB and the PR in other cell types (Kalkhoven *et al*., 1996). Our results have indicated that this negative relationship also exists within amnion cells. IL-1β stimulation causing NF-κB activation in pre-labour cells was able to repress progesterone-dependent transcription even in the presence of excess PR. In the reverse experiment, low levels of NF-κB transcriptional activity in pre-labour amnion cells were repressed by the PR.

In the context of human labour, it is the repression of PR function by NF-κB, rather than the converse, which is of greatest physiological importance. Studies of the mutual negative interaction between NF-κB and the steroid receptor family have suggested the involvement of both direct interactions between NF-κB and steroid receptors and competition for common co-factors such as CREB binding protein (CBP) and steroid receptor co-activator 1 (SRC-1) (De Bosscher *et al*., 1997; Dumont *et al*., 1998; Sheppard *et al*., 1999).

We have shown constitutive activity of NF-κB in amnion cells in association with human labour. This NF-κB activity increases the expression of COX-2 and appears to contribute to the 'functional' progesterone withdrawal through an interaction with PR. Other labour-associated inflammatory mediators such as IL-1β, IL-6 and IL-8 have also been shown to be up-regulated by NF-κB. NF-κB therefore plays a central role in the biochemistry of human labour. We have identified a single mechanism which could underlie both increased prostaglandin and inflammatory cytokine synthesis and a withdrawal of the classical 'progesterone block'. The increase in NF-κB activity in amnion appears to occur near to, or at the time of, labour. It is possible that this represents a watershed point at which labour becomes inevitable. Understanding how NF-κB activity is regulated within the uterus may provide new insights into the control of human labour.

Acknowledgements

This study was funded by MRC and Wellbeing. We wish to thank Pierre Chambon and Birgit Gellerson for proving DNA constructs.

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Received on September 13, 2000; accepted on March 9, 2001