Expression and Deoxyribonucleic Acid-Binding Activity of the Nuclear Factor κB Family in the Human Myometrium during Pregnancy and Labor

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In humans, the factors that govern the switch from myometrial quiescence to coordinated contractions at the initiation of labor are not well defined. The onset of parturition is itself associated with increases in a number of proinflammatory factors, many of which are regulated by the nuclear factor κB (NF-κB) family of transcription factors. The expression and DNA-binding activity of NF-κB in the myometrium during gestation and parturition were examined. Levels of c-Rel, p50, and p105 NF-κB species were dramatically reduced in pregnant myometrium compared with nonpregnant (NP) controls, whereas expression of the RelA subunit remained uniform. Importantly, during labor, expression of all subunits was observed to be significantly reduced in all myometrial samples studied relative to NP levels. Moreover, for RelA, c-Rel, and p50 subunits, there was a gradient of expression between laboring upper (corpus) and lower uterine segment myometrium. No RelB or p52 subunits could be detected. EMSAs identified changes in NF-κB subunit composition in the myometrium during pregnancy and labor, with p50 homodimers predominant in NP tissues being replaced with RelA:p50 heterodimers in pregnant and laboring samples. Significantly, RelA was observed to be phosphorylated at serine-536, implicating the involvement of the phosphatidylinositol-3-kinase/AKT pathway in NF-κB function in the myometrium. (J Clin Endocrinol Metab 89: 5683-5693, 2004)
opportunities therefore arise for highly specific gene expression in response to a given stimulus.

The role of NF-κB in human parturition has focused on regulation of COX-2 and IL-8 in amnion cells (40–44). Although such studies provide vital information about the regulation of individual amnion-expressed genes associated with labor, they do not examine myometrium and concentrate on a limited number of characterized promoters. Soloff et al. (45) recently demonstrated the importance of the RelA subunit in controlling COX-2 and IL-8 expression in cultures of primary human myometrial myocytes (45) using the chromatin immunoprecipitation (ChIP) assay. The spatio-temporal regulation of other NF-κB subunits in the myometrium during pregnancy and labor, however, is not known. Because the myometrium is exposed to a complex milieu of cytokines, chemokines, and other signaling factors, we hypothesized that other NF-κB subunits are involved in myometrial smooth muscle gene expression. Consequently, the purpose of the present study was to employ Western blotting, coimmunoprecipitation, and EMSAs to characterize expression and DNA-binding activity of members of the NF-κB subunit family in the myometrium sampled from the upper (corpus) and lower uterine segments during pregnancy and labor.

Patients and Methods

Selection of patients and tissue collection

All women were recruited from the Department of Obstetrics and Gynaecology at the Royal Victoria Infirmary, Newcastle-upon-Tyne. This study received approval from the Newcastle and North Tyneside Health Authority Ethics Committee, and all patients gave informed consent.

Nonpregnant (NP) myometrium. Myometria were obtained from NP premenopausal women (n = 14; age, 32–46) undergoing hysterectomy for benign gynecological conditions. The samples were taken in theater immediately after the removal of the uterus. The uterus was incised longitudinally, and samples of myometrium were taken from the middle of the uterine wall, with care being taken to allow generous clearance margins from the serosal and endometrial surfaces. Samples obtained in both the follicular and luteal phases of the cycle were used.

Term pregnant (P) myometrium. Upper and lower uterine segment myometrial samples were obtained from healthy women undergoing elective cesarean section at term (n = 14; age, 16–43; gestation, 37–40 wk).
The indications for section were: breech presentation, previous cesarean section, or bad previous obstetric outcome. Excluded from this group were women whose cervixes had dilated beyond 2 cm or who were experiencing regular painful uterine contractions. Patients who had had prostaglandin gel administered or whose amniotic membranes were not intact were also excluded. The samples were obtained immediately after the delivery of the placenta and membranes before the closure of the uterine cavity. Samples from the upper uterine segment were taken under direct vision using Wolf biopsy forceps introduced into the uterine cavity through the incision (46). The forceps were pushed through the decidual layer and into the myometrium. Eight separate myometrial biopsies were taken from individual patients, each from a nonplacental bed site (as determined by manual palpation before the delivery of the placenta). Samples from the lower uterine segment were taken from the upper lip of the incision through the lower uterine segment using toothed biopsy forceps to grasp the myometrium from between its serosal and decidual layers, then curved scissors to sample it.

**Term spontaneously laboring (SL) myometrium.** Upper and lower segment myometrial samples were obtained from women admitted in spontaneous labor (as defined as the onset of painful regular uterine activity resulting in the progressive and serial dilatation of the cervix beyond 3 cm) undergoing emergency cesarean section at term (n = 14; age, 16-41 yr; gestation, 37-42 wk). Indicators for section were failure to progress and fetal distress. Women who had their labor induced or augmented before reaching 3 cm were excluded from the study. Upper and lower segment biopsies were obtained in a manner similar to that used for women undergoing elective cesarean section. All myometrial samples were snap frozen at the time of collection using liquid nitrogen-cooled isopentane, and then stored at -70°C.

**Preparation of myometrial homogenates.** Homogenates of myometrial tissue from NP, P, and SL patients were prepared as detailed by MacDougall et al. (6). Total protein was quantified using the Bio-Rad (Hercules, CA) DC protein assay kit, following manufacturer’s guidelines. Equal loading of gels was verified by probing each blot for the Gβ subunit, levels of which remain constant during pregnancy and labor (4, 47).

**Western immunodetection analysis.**

NF-kB expression was examined using Western analysis with immunoblots probed for each NF-kB subunit essentially as detailed by Chapman et al. (31). After SDS-PAGE, resolved proteins were then electroblotted onto nitrocellulose (Sigma, St. Louis, MO). The gel and membrane were first briefly soaked in Toubin transfer buffer (25 mM Tris, 192 mM glycine, and 20% [vol/vol] methanol) and then blotted for 90 min at 9 V (constant current, 90 mA). Equal loading of gels was verified by probing each blot for the Gβ subunit, levels of which remain constant during pregnancy and labor (4, 47).

**EMSA**

EMSAs were performed using 100 μg crude myometrial homogenate in a 20-μl reaction vol consisting of 20 mM Tris (pH 7.6), 200 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, 1 μg poly (dl-cd:dl-dc), and approximately 0.1 ng α-32P-labeled double-stranded HIV 3′ long terminal repeat (LTR) xB site (5′-GATCCGGTTGGGACTTCCAGG C3′; xB site in boldface) or the MHC H2-β2 site (5′-GATCCGGCTGGGAAATC- CCC AGGGC-3′; xB site in boldface). Homogenates were preincubated for 5 min at room temperature with reaction buffer minus probe to assess the specificity of binding. 100 ng (1000-fold excess) of the respective cold (unlabeled) xB oligonucleotides was included with the labeled probe in each homogenate. For supershift analyses, antisera were included in the preincubation step; RelA supershifts used the sc-109 antisemur. After this time, labeled probe was added and incubations continued for a further 15 min at room temperature. DNA:protein complexes were then resolved using 1× Tris-glycine-EDTA (25 mM Tris, pH 8.0, 190 mM glycine, and 1 mM EDTA)-4% nondenaturing polyacrylamide gels.

**Coimmunoprecipitation**

To determine the nature of myometrial proteins that bound to endogenous NF-kB subunits, coimmunoprecipitation analysis was performed. Briefly, 400–600 μg crude myometrial homogenates were diluted 5-fold in incubation buffer (20 mM HEPES, pH 7.9, 75 mM KCl; 2.5 mM MgCl2; 0.1% [vol/vol] Nonidet P-40; 1.0 mM NaVO3; 0.5 mM phenylmethylsulfonyl fluoride; 1 μg/ml leupeptin; 1 μg/ml aprotinin; and 1 μg/ml E64), and then 1 μg of either anti-RelA (sc-109) or control IgG was then added to each sample. Samples were then incubated at 4°C for 1.5 h with constant agitation. After this time, 10–15 μl bed vol of protein-G agarose Fast Flow (Upstate Biotechnology, Inc.; no. 16–266) was added and the samples incubated for a further hour at 4°C. Finally, the beads were washed in 3× 500 μl vol of incubation buffer and then resuspended in 15–20 μl sodium dodecyl sulfate-loading buffer. Samples were then resolved by 10% SDS-PAGE and then subjected to Western analysis.

**Statistical analysis**

Data were compared using an unpaired, two-tailed t test; P < 0.05 was considered statistically significant. All results are expressed as the mean ± sem.

**Results**

**Temporal expression of NF-kB subunits in lower segment myometrial homogenates from NP, P, and SL women.** Employing specific antibodies in Western immunodetection, expression of the NF-kB subunits RelA, c-Rel, p105, p50, and p100 were determined in NP, P, and SL tissues biopsied from the lower uterine segment. Expression of RelA between NP and P tissues remained uniform but was significantly reduced in SL samples (Fig. 2A). Expression of both c-Rel (Fig. 2B) and p105 (Fig. 2C) was observed to be sequentially decreased in P and SL samples in comparison with NP controls. Expression of p50 (Fig. 2D) and p100 (Fig. 2E) subunits was substantially decreased in P tissues compared with NP controls but was not decreased any further in SL samples. To ensure equal loading of all samples, blots were reprobed with the Gβ-subunit antibody (Fig. 2F). Both Rel B and p52 remained undetected in any myometrial investigated (data not shown).

**Spatial expression of NF-kB subunits in upper (corpus) and lower uterine segment myometrial homogenates from P and SL women.** To determine whether topographical distributions of NF-kB subunits occurred in different regions of the uterus...
during pregnancy and labor, Western immunodetection with RelA, c-Rel, p105, p50, and p100 antibodies was performed on myometrial tissue homogenates sampled from the upper (corpus) and lower uterine segments from P and SL women. Interestingly, of the five NF-κB subunits studied, expression of RelA (Fig. 3A), c-Rel (Fig. 3B), and p50 (Fig. 3D) was observed to be significantly decreased in lower, compared with upper, uterine segment myometrium sampled from SL women; whereas uniform expression was observed for p105 (Fig. 3C) and p100 (Fig. 3E) species in upper- and lower-segment SL myometrial samples.

A change in NF-κB dimer composition occurs between NP, P, and SL in lower-segment myometrial homogenates

Western analysis of total levels of cellular NF-κB proteins does not provide an accurate reflection on the actual level of NF-κB that is competent to bind DNA and hence modify expression of particular genes. Therefore, to circumvent this problem, EMSAs were performed to assess whether there was any change in the level of NF-κB:DNA binding among NP, P, and SL samples. Using the HIV LTR 3′-κB site oligonucleotides, four protein:DNA complexes could be resolved in NP, P, and SL samples (Fig. 4A; labeled I–IV). Complexes I and II were predominant in NP samples and greatly reduced in those from P and SL homogenates. Complex III was present in all samples and was seen to become more dominant in P and SL extracts. Complex IV was predominant in NP tissues and virtually absent in P and SL homogenates. Furthermore, the overall level of total NF-κB:DNA binding was seen to decrease in P and SL compared with NP samples, thus reflecting the immunoblotting data in Fig. 2. Equal amounts of myometrial homogenates (100 μg) were used in each EMSA. Equal loading of samples was confirmed by probing separate immunoblots for Gβ (Fig. 4A, lower).

To determine the identity of the complexes observed in Fig. 4A, supershift analysis, using a range of anti-NF-κB antisera, was performed (Fig. 4B). The upper complex, band I, removed with cold excess HIV-κB oligonucleotides, was also removed with both nonspecific IgG and various NF-κB antisera, suggesting that it represented nonspecific protein:DNA binding. The identity of complex II remains unclear.
although it was exclusive to NP homogenates. It may represent RelA and/or c-Rel homo- and heterodimers (RelB does not homodimerize), although it was difficult to observe any supershift with these NF-κB antisera, suggesting that it may also represent nonspecific binding.

Complex III likely represents heterodimers of RelA:p50 and RelB:p50 (Fig. 4B, band III) because, in NP samples, there are very slight shifts of this complex when these antisera are included in the binding reactions. In P and SL homogenates RelA:p50 heterodimers are seen to be the predominant NF-κB complex in myometrium (Fig. 4B, band III). Complex IV represents p50 homodimers. Significantly, these homodimers are the major NF-κB proteins present in NP samples (Fig. 4A, band IV), being very much reduced in both P and SL homogenates. No supershifts were seen in NP tissues with either c-Rel or p52 antisera. Furthermore, no supershifts were observed in P and SL samples when RelB, c-Rel, or p52 antisera were employed. Together, these observations suggest that there is a major change in NF-κB dimer composition among NP, P, and SL tissues. Importantly, whereas there is an overall reduction in total NF-κB:DNA binding in P and SL samples compared with NP, the actual level of RelA:p50:DNA binding is seen to increase in P and SL homogenates compared with NP.

Although all NF-κB subunits generally bind to the HIV-3’LTR κB site with similar affinities in vitro (low picomolar) (48), the p52 subunit binds with an approximately 15-fold lower affinity than the similar p50 protein. Although no p52 was observed in NP, P, or SL tissues using Western analysis, it was important to determine whether any p52 binding could be detected using the more sensitive EMSA and supershift studies. This second round of EMSAs used the H2-κB oligonucleotides. This site is palindromic and binds p50 and p52 with similar affinity (48). A pattern of binding similar to that seen for the HIV-3’ LTR κB site was observed when NP, P, and SL myometrial homogenates were incubated with the H2-κB site (Fig. 5A, complexes I-IV). No p52 protein, however, was detected when supershift analysis
was performed (Fig. 5B), suggesting that this subunit does not play a part in myometrial smooth muscle gene regulation. RelB:p50 supershifts in NP tissues are seen as a reduction in intensity of complex III. Gβ/H9252-immunodetection illustrated that all samples were equally loaded (Fig. 5A, lower).

**RelA coimmunoprecipitates with the catalytic Ca-subunit of PKA (PKAcα) and IκBα and is phosphorylated at serine-536 in NP and P lower uterine segment myometrium**

Because RelA regulates the expression of the other NF-κB proteins (10), the decrease in RelA observed in SL samples would thus account for the decreases in the other NF-κB family members. It does not, however, explain why there is reduction in intensity of complex III. Gβ-immunodetection illustrated that all samples were equally loaded (Fig. 5A, lower).

**RelA is phosphorylated at many other sites, and those that have been mapped include Ser 529 (52) and Ser 536 (53).** All these phosphorylations have been reported to be essential for RelA transactivation. Therefore, it was of interest to determine whether RelA in NP and P myometrial homogenates...
was phosphorylated on serine residues other than Ser 276. RelA was immunoprecipitated from NP and P myometrial homogenates as detailed in Patients and Methods. Figure 6C illustrates that, in both NP and P myometrial tissues, RelA is phosphorylated on serine-536. The significance of these observations is addressed below.

Discussion

Myometrial NF-κB is regulated in a spatio-temporal fashion

We have examined the expression of members of the NF-κB family of transcription factors in NP, P, and SL myometrial tissues. Our findings illustrate, for the first time, that there is a specific pattern of expression for each NF-κB subunit in the myometrium during pregnancy and labor. RelA, c-Rel, p100, p105, and p50 were all detected at high levels in NP tissues. With the exception of RelA, significant reductions were observed in all other NF-κB subunits in both P and SL samples compared with NP controls; whereas no differences in the levels of RelA were seen between NP and P myometrial samples. A significant reduction in the level of RelA, however, was observed in SL compared with P tissues. Using Western analysis and EMSA, no p52 could be detected in any myometrial samples investigated, suggesting that this subunit may not be critical in regulating myometrial gene expression and activity. The p52 antiserum specifically recognizes the same epitope in the p100 precursor, which was detected, suggesting that the antiserum was functioning appropriately. Importantly, the decrease in p100 observed in P and SL homogenates did not cause a concomitant rise in levels of the p52 subunit, suggesting that that processing of p100 to p52 was not occurring in these tissues.

Although Western analysis failed to detect RelB in any myometrial tissues, EMSA and supershift experiments identified a slight shift in NP homogenates with RelB antiserum. An explanation for this dichotomy is that the RelB antiserum employed may predominantly recognize native, folded protein such as that in the EMSA, whereas that bound to the immunoblots would be mostly denatured with the concomitant loss of recognizable epitopes.

Interestingly, there appeared to be a topographical distribution of myometrial RelA, c-Rel, and p50 subunits within different regions of the uterus during labor, because levels of these proteins were significantly reduced in lower, compared with upper, uterine segment biopsies in SL women. It is well established that the upper and lower regions of the uterus govern contractility and dilatation, respectively, during labor. We have previously reported the differential expression of myometrial COX-1 and -2 within different uterine regions at term and during labor (7), and this is consistent with data describing spatio-temporal expression of other contractile-
associated proteins (7, 54, 55). Because COX-2 expression has been shown to be regulated in the myometrium during gestation and parturition by the NF-κB pathway (40–44), the possibility exists that the spatial expression of RelA, c-Rel, and p50 subunits may be involved in this process as well as modulating the differential expression of other key regulatory genes.

Modification of NF-κB dimer composition between NP, P, and SL tissues

EMSA were used in the present study to assess the level of NF-κB DNA binding in each tissue. Data presented in Figs. 4 and 5 illustrate that there are NF-κB proteins present in each homogenate capable of binding to defined, consensus κB sites. A decrease in NF-κB p50:DNA binding concomitant with an increase in RelA:p50 DNA binding in P and SL homogenates was observed, compared with NP samples. In NP homogenates, NF-κB p50 DNA binding was predominant, with a very small amount of RelA:p50 also being observed. At present, the significance of this change in NF-κB dimer composition is unclear. In simplistic terms, p50 homodimers are generally seen to be transcriptional repressors (56, 57), whereas RelA:p50 heterodimers are generally associated with transcriptional activation (16). Therefore, it is tempting to speculate that the observed change in dimer composition from inhibitory p50 complexes to stimulatory RelA:p50 subunits occurs to specifically modulate changes in expression of genes critical for myometrial activation and contraction.

This simplistic view, however, does not consider the natural promoter contexts under which the various NF-κB dimers would be acting. This is because EMSAs only demonstrate protein:DNA interaction; any selectivity provided when the site is within its native promoter is generally lost (23). Furthermore, EMSAs do not provide information detailing whether the DNA-bound factor causes repression or activation of gene expression. An example to illustrate this point is the repression of antiapoptotic gene expression by RelA:p50 heterodimers in response to UV stimulation (58, 59). In these studies, Campbell et al. (58), illustrated that UV stimulation of an osteosarcoma cell line (U2-OS) caused RelA:p50 DNA binding to the HIV 3′ LTR κB site as judged by EMSA (58). Such NF-κB complexes, however, were transcriptionally inert and served to actually repress antiapoptotic gene expression by recruiting HDAC-1, 2, and 3 to the appropriate promoter regions (59). Thus, in the context of our study employing myometrial homogenates, whereas we were clearly able to illustrate the change in dimer composition between NP, P, and SL samples, we were unable to determine the nature of NF-κB-regulated genes or whether they were activated or repressed in a NF-κB-dependent manner.

Temporal changes in NF-κB subunit composition on NF-κB-regulated promoters permits a fine-tuning of the transcriptional response, ensuring that the gene is expressed at the correct level for the appropriate length of time (23). For example, in mouse dendritic cells, the macrophage-derived chemokine promoter is activated by RelA-containing dimers in response to lipopolysaccharide stimulation. Over a period of hours, RelB then replaced RelA, whereas transcription from the macrophage-derived chemokine promoter remained unaffected (23). It is likely that such fine-tuning also
occurs in the human myometrium as it progresses initially from a NP, cycling state to a P state, where the lower segment is contracted and the upper segment relaxed, and then finally into labor, where the lower segment relaxes and the upper segment contracts. Using myometrial homogenates would not permit such a rigorous analysis of dimer exchange as described by Saccani et al. (23) above. As such, addressing the functional specificity of myometrial NF-κB gene regulation in vitro is the basis of ongoing work in our laboratory.

**RelA phosphorylation**

It is well documented that RelA, c-Rel, p105, and p100 are subject to constitutive phosphorylation in unstimulated cells (reviewed in Ref. 24). In the present study, it would be impossible to determine the numbers of stimulated and unstimulated cells in the myometrial biopsy before homogenization. As such, we assume that there would also be a basal level of constitutive NF-κB phosphorylation in myometrium.

RelA was seen to specifically associate with both the PKAcα subunit and IkBa in both NP and P myometrial homogenates. The presence of IkBa in this complex fully accounts for the inability to detect serine-276 phosphorylation of RelA induced by PKA (50). The fact that some myometrial PKAcα is sequestered into an inactive complex is of interest. Normally, PKAcα is held in an inactive complex by associating with two regulatory subunits. During quiescence, Gαi stimulation causes increases in intracellular levels of cAMP (3). cAMP, in turn, binds to, and induces dissociation of, the regulatory proteins from PKA, thereby releasing the catalytically active PKAcα subunits (3–6). Upon induction of labor, however, there is down-regulation of this pathway (4). RelA-associated PKAcα is not bound to the normal regulatory subunits but is, instead, bound by IkBa, which serves a similar regulatory function over PKAcα, maintaining it in an inactive state until the cell is exposed to a RelA-inducing stimuli (e.g. IL1β or TNFa; 49, 50). Therefore, we postulate that, during quiescence, a distinct pool of RelA is associated with IkBa-regulated, cAMP-independent PKAcα. Upon induction of labor, however, the regular PKA pathway is repressed (4–6). RelA, however, could still undergo rapid PKAcα-mediated phosphorylation in response to the appropriate stimuli, to then up-regulate various contractile-associated proteins, such as COX-2, which would propagate pro-labor signals.

The importance of serine-536 phosphorylation on RelA in both NP and P homogenates is unclear. Serine-536 phosphorylation is induced directly by both IKKa and IKkB as a result of IL-1-induced phosphatidylinositol-3-kinase/AKT activation (60–62). In this pathway, IKkB-induced phosphorylation of IkBa is still required to permit RelA to dissociate from its inhibitor. IKKa- and IKkB-induced phosphorylation of serine-536 of RelA, however, is absolutely required if RelA is to attain its full transactivation potential. Taken together, our data suggest that the association of RelA with PKAcα and the phosphorylation of RelA at serine-536 may reflect a mechanism by which different intracellular signaling pathways cross-talk with each other, thus facilitating the regulation of distinct myometrial functions.

**Reduction in levels of RelA**

It was not possible to determine what caused the decrease in levels of myometrial RelA during labor. Chapman et al. (31) have described the formation of an N-terminal truncation of RelA (RelA p37) upon coexpression with either c-Myc or a constitutively active form of Ras in monolayers of human embryonic kidney 293 cells (31). The RelA antisera used in the present study specifically recognized the C terminus of RelA. When the N-terminal antisera (sc-109) was used, however, no RelA p37 was observed, suggesting that this mechanism of RelA degradation was not used in myometrium (data not shown). It is plausible that proteosomal degradation accounts for the reduction in RelA in SL homogenates. Interestingly, Perissi et al. (63) recently reported a mechanism by which precise control over the switch from gene expression to gene repression could occur (63). In this study, two related proteins, termed TBL1 and TBLR1, served as specific adaptors for the recruitment of ubiquitin conjugating/195 proteosome complex to various steroid and NF-κB responsive promoters. Having the proteosome both physically and exclusively recruited to a subset of RelA-regulated promoters that maintain quiescence would permit the specific degradation of RelA on those quiescence-associated promoters as parturition began. This mechanism would not affect those RelA-regulated genes vital for parturition, such as COX-2 and IL-8, whose activity would then be stimulated, allowing normal parturition to ensue. Such a hypothesis would be in keeping with our observations that, whereas the total level of RelA protein decrease in SL myometrial homogenates, the actual level of RelA p50 DNA binding is seen to increase.

In conclusion, the salient points arising from this study are:

1) There is a switch in NF-κB subunit composition between NP, P, and SL tissues. 2) Although a decrease in RelA levels was observed in Western analysis, the level of RelA p50 DNA binding actually increases. 3) RelA associates with PKAcα and also undergoes phosphorylation at serine-536, implicating the involvement of both the PKA and phosphatidylinositol-3-kinase/AKT pathways in regulating distinct aspects of human myometrial NF-κB function.

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