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Article Title: The Onset of Labor Alters Corticotropin-Releasing Hormone Type 1 Receptor Variant Expression in Human Myometrium: Putative Role of Interleukin-1ß
Year of publication: 2007
Link to published article:
http://dx.doi.org/10.1210/en.2007-0095
Publisher statement: None

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Endocrinology 2007 148:3205-3213 originally published online Apr 12, 2007; , doi: 10.1210/en.2007-0095

0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(7):3205–3213

doi: 10.1210/en.2007-0095

The Onset of Labor Alters Corticotropin-Releasing Hormone Type 1 Receptor Variant Expression in Human Myometrium: Putative Role of Interleukin-1β

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CRH targets the human myometrium during pregnancy. The efficiency of CRH actions is determined by expression of functional receptors (CRH-R), which are dynamically regulated. Studies in myometrial tissue biopsies using quantitative RT-PCR demonstrated that the onset of labor, term or preterm, is associated with a significant 2- to 3-fold increase in CRH-R1 mRNA levels. Detailed analysis of myometrial CRH-R1 mRNA variants showed a decline of the pro-CRH-R1 mRNA encoding the CRH-R1β variant during labor and increased mRNA levels of CRH-R1d mRNA. Studies in myometrial cells identified IL-1β as an important regulator of myometrial CRH-R1 gene expression because prolonged treatment of myometrial cells with IL-1β (1 ng/ml) for 18 h induced expression of CRH-R1 mRNA levels by 1.5- to 2-fold but significantly attenuated CRH-R1β mRNA expression by 70%. In contrast, IL-1β had no effect on CRH-R1d mRNA expression. Studies using specific inhibitors suggest that ERK1/2, p38 MAPK, and downstream nuclear translocation of nuclear factor-κB mediate IL-1β effects on myometrial CRH-R1 gene. However, the increased CRH-R1 mRNA expression was associated with a dampening of the receptor efficacy to activate the adenylyl cyclase/cAMP signaling cascade. Thus, our findings suggest that IL-1β is an important regulator of CRH-R1 expression and functional activity, and this interaction might play a role in the transition of the uterus from quiescence to active contractions necessary for the onset of parturition. (*Endocrinology* 148: 3205–3213, 2007)

DURING GESTATION, THE uterus remains in a state of quiescence while the fetus matures. At term, delivery is facilitated by cervical dilatation and a switch of the uterus from quiescence to a state of coordinated contractility (1). In humans, the mechanism of parturition involves multiple pathways, and some are regulated by "stress" peptides, in particular placental CRH. The plasma concentrations of CRH increase exponentially as pregnancy progresses toward labor and may act as a predictor of the duration of human gestation (2).

The precise biological function of CRH and the family of CRH-related peptides, urocortins, during pregnancy are not well defined, but they appear to play important roles in regulation of myometrial contractility (3). The human myometrium expresses a plethora of CRH and CRH-related peptides and their respective receptors R1 and R2 (4). Current evidence suggests that CRH-R1- and CRH-R2-activated signaling pathways might exert distinct actions in the regulation of myometrial contractility (4). The myometrial CRH-Rs exhibit increased affinity for CRH during pregnancy (5) and, on agonist activation, stimulate cAMP and cGMP production and nitric oxide synthase up-regulation (6), thus promoting myometrial quiescence, possibly though activation of CRH-R1 receptors. Recent isometric contractility studies in myometrial tissue strips have confirmed the ability of CRH to relax the human pregnant myometrium (7). In contrast, CRH-R2-specific agonists such as urocortin-II can activate ERK1/2 and RhoA pathways that actively promote myometrial contractility (8). Furthermore, progression of human pregnancy toward term and active labor is associated with activation of intracellular mechanisms involving uterotonin-activated protein kinase C that reduce the myometrial responsiveness to CRH, an effect mediated through phosphorylation and desensitization of specific CRH-R variants (9, 10).

The human CRH-R1 gene spans over a 50.3-kb region and contains 14 exons (11); translation of all exons results in a human-specific, 444-amino acid seven-transmembrane domain protein receptor, termed CRH-R1β, which exhibits impaired agonist binding and signaling properties. Deletion of exon 6 results in expression of CRH-R1α mRNA, which is the main functional CRH-R1 receptor variant and contains 415 amino acids. Therefore, the CRH-R1β can be considered as a "pro-CRH-R1" receptor variant. The CRH-R1 gene appears to be subject to significant alternative splicing, and a growing number of CRH-R1 mRNA splice variants have been described, termed R1c–R1n (11). All of these variants have exon 6 spliced out together with other deletions. At least four distinct CRH-R1 mRNA variants (α, β, c, and d) have been

First Published Online April 12, 2007

Abbreviations: C_T, Threshold cycle; DAPI, 4′,6′-diamidino-2-phenylindole; F-actin, filamentous actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IKK, IκB kinase; MEK, MAPK kinase; PDE, phosphodiesterase; PGE_2, prostaglandin E_2; PTGS2, prostaglandin-endoperoxide synthase 2; R, receptor; TBS, Tris-buffered saline; TBS-T, TBS containing 0.1% Tween 20.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

identified in human myometrium during pregnancy (12); progression toward term is associated with altered expression of myometrial CRH-Rs and the appearance of CRH-R1 variants such as the R1d, with reduced signaling abilities (13). This might have important functional consequences by dampening tissue responsiveness to CRH actions.

The mechanisms regulating myometrial CRH-R1 expression are not well understood. The CRH-R1 promoter contains, among others, nuclear factor-κB (NF-κB) putative recognition elements (κBs) elements (14). Accumulating evidence points toward a role for NF-κB and its major activator IL-1β in the physiology and pathophysiology of labor (15), because myometrial IL-1β and NF-κB activity increases with labor onset and is central to multiple prolabor pathways by stimulating prostaglandin-endoperoxide synthase 2 (PTGS2) expression and prostaglandin (PG) synthesis (16). In this study, we examined the levels of expression of CRH-R1 and specific CRH-R1 variants by using quantitative RT-PCR analysis from myometrial biopsies obtained preterm and term, before and after the onset labor as well as the regulation of CRH-R1 expression and activity by IL-1β in human pregnant myometrial cells.

Materials and Methods

Subjects and myocytes culture preparations

Pregnant myometrial biopsies were obtained from women undergoing elective cesarean section preterm (30–35 wk of gestation; n = 12) or at term (>37 wk of gestation; n = 12) before (n = 6) or after (n = 6) the onset of labor for nonmaternal problems. Active labor was demonstrated by regular uterine contractions and ripe cervix, with dilation more than 4 cm (range of 4–7 cm; mean of 5.4 cm). The biopsy site was standardized to the upper margin of the lower segment of the uterus in the midline. This provides the closest approximation to the upper segment of the uterus. The biopsies were immediately snap frozen in liquid nitrogen and subsequently stored at −70 C until use or processed immediately for myocyte cell culture as described previously (10). The age range was 25–35 yr for both groups. Ethical approval was obtained from the local ethical committee, and informed consent to the study was obtained from all patients.

The myometrial smooth muscle cells were cultured in DMEM containing 10% fetal calf serum, 1% L-glutamine, 200 IU/ml penicillin, 200 mg/ml streptomycin, at 37 C in a humidified atmosphere of 95% air and 5% CO_2 until confluency. Cells were trypsinized in 0.25% trypsin containing 0.02% EDTA in Hanks' balanced salt solution and cultured in 12-well plates for protein and in six-well plates for mRNA extraction. At 18 h before treatments, the cells were transferred in DMEM without L-glutamine, fetal calf serum, and antibiotics. The following day, cells were treated with IL-1β (1 ng/ml) for various time intervals (2–18 h). In some experiments, cells with pretreated with or without specific inhibitors, as follows: 10 μM U0126 [1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene] (2 h) [U0126 inhibits both active and inactive MAPK kinase (MEK1/2)], 10 μM SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole] (1 h) (SB203580 is a selective inhibitor of p38 MAPK, which acts by competitively inhibiting ATP binding), 25 μM IκB kinase (IKK) inhibitor II, Wedelolactone (4 h) (IKK inhibitor II is a selective and irreversible inhibitor of IKKα and β kinase activity; it inhibits NF-κB-induced gene transcription by blocking the phosphorylation and degradation of IκBα). Proteins and RNA were harvested as described below. For confocal microscopy experiments, serum-deprived cells were stimulated with 1 ng/ml IL-1β for 30 and 60 min. The purity of myometrial muscle cells was assessed by immunocytochemical staining for α-actin with mouse monoclonal antibody specific for smooth muscle α-actin (Sigma, Gillingham, UK). Confluent cells up to third passage were used; no significant difference between results were obtained with cells from individual passages and cells obtained from different myometrial biopsies.

Chemicals

CRH was purchased from Bachem (Merseyside, UK). IL-1β and the inhibitors (U0126, MEK1/2 inhibitor; SB203580, p38 inhibitor; and IKK inhibitor II, Wedelolactone) were from Merck Biosciences (Nottingham, UK). Antibodies (CRH-R, p65, IκBα, and PTGS2) were from Santa Cruz Biotechnology (Santa Cruz, CA); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from BioGenesis (Bournemouth, UK); secondary antibodies were from DakoCytomation (Eye, UK); Alexa Fluor594, Alexa Fluor488-phalloidin, and *Slowfade* gold antifade reagent with 4′,6′-diamidino-2-phenylindole (DAPI) were from Invitrogen (Carlsbad, CA). Vectashield Hard Set TM mounting medium for fluorescence was from Vector Laboratories (Burlingame, CA). Mounting solution without DAPI was from Vector Laboratories (Peterborough, UK). Cell culture media were from Invitrogen. RNasin, avian myeloblastosis virus, random hexamers, and oligo-dTTP were from Promega (Madison, WI), and dNTPs, *Taq* polymerase, and DNA ladder were purchased from Bioline (London, UK). SYBR Green I and PCR mixture for quantitative PCR was from BioGene (Kimbolton, UK). Primers were purchased from TANG (Gateshead, UK). All other chemicals were purchased from Sigma.

RNA extraction and real-time and conventional RT-PCR

RNA extraction was performed by using GeneElute Mammalian Total RNA kit (Sigma), according to the instructions of the manufacturer. The purity and concentration of total RNA was determined spectroscopically. RNA, 500 ng, was used for cDNA synthesis, in a total volume of 50 μl RT reaction. Total RNA was heated up to 70 C for 5 min before the addition of random hexamers (Promega) and reverse transcriptase containing an RNase inhibitor (Fermentas, York, UK). The reaction mixture was incubated at 42 C for 60 min, followed by an additional 10 min incubation at 70 C.

Quantitative (real-time) PCR of PTGS2 was performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany). The PCR was performed in a 10 μl reaction mixture containing 5 μl of PCR 2× Mastermix with 2 mM $MgCl_2$, 0.5 μl of Light Cycler DNA Master SYBR Green I, 1 μl of each primer (2 ng/μl), and 1 μl of cDNA. The PCR protocol consisted of a denaturation step at 95 C for 15 sec, followed by 40 cycles of amplification at 95 C for 5 sec, 58 C for 10 sec, and 72 C for 15 sec, and finally by a melting-curve analysis step at 95 C for 0 sec, 56 C for 15 sec, and 99 C for 0 sec. For analysis, quantitative amounts of gene of interest were standardized against the housekeeping gene β-actin. The sequence of PTGS2 mRNA primers used were as follows: forward, 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′; and reverse, 5′-AGATCATCTCTGCCTG AGTATCTT-3′. The β-actin primers were as follows: forward, 5′-AAGAGAGGCATCCTCACCCT-3′; and reverse, 5′-TACATGGCTGGGGTGTTGAA-5′. As negative controls, preparations lacking RNA or reverse transcriptase were used. RNA expression was tested in four independent experiments. The mRNA levels were expressed as a ratio, using the "ΔΔ method" for comparing relative expression between treatments. The PCR products were sequenced in an automated DNA sequencer (Department of Biological Sciences, The University of Warwick, Warwick, UK), and the sequence data were analyzed using Blast Nucleic Acid Database Searches from the National Center for Biotechnology Information.

Quantitative PCR on a TaqMan Gene Expression Assay (Applied Biosystems, Warrington, UK) was also used to determine expression levels of various CRH-R1 mRNA variants in human gestational myometrium and human primary myometrial cells. Briefly, four sets of oligonucleotide primers and four TaqMan probes specific for various human CRH-R1 mRNAs were designed from the GenBank database by *mySciences* (Applied Biosystems). The first primers/probe combination used amplified a sequence overlapping exons 8–9, which is present in all known CRH-R1 variants detected in the human myometrium, R1α, R1β, R1c, and R1d (12). Therefore, the PCR product reflected total CRH-R1 mRNA variant expression and was designated as $CRH\text{-}R1_T$. In addition, primers and fluorescent probes specific for exon 6 (found in R1β mRNA variant only) were designed, and the PCR amplification product was designated as CRH-R1β. Similarly, primers and probes overlapping exons 2–4 and 12–14 were designed to amplify sequences specific for CRH-R1c and CRH-R1d (Fig. 1). Real-time RT-PCRs were performed using the ABI PRISM 7000 Sequence Detection System (Ap-

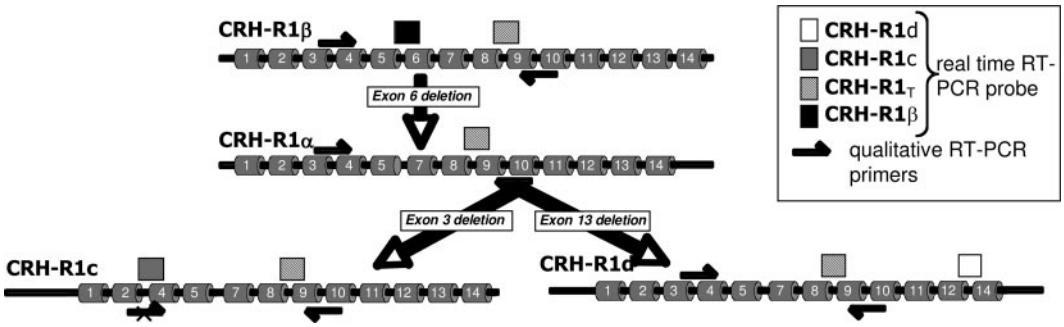

FIG. 1. Schematic diagram of CRH-R1 gene splicing pattern and the annealing position of PCR primers and real-time PCR probes relative to the structure of CRH-R1 alternative spliced variants. *Arrows* indicate annealing position of primers. The qualitative RT-PCR forward primer was designed to span across exons 3 and 4; thus, annealing to CRH-R1c sequence was not possible.

plied Biosystems) in a total volume of 30 μl reaction mixture following the protocol of the manufacturer, using the SYBR Green Universal 2× PCR Master Mix (Applied Biosystems) and 0.1 μM of each primer using the dissociation protocol for the amplification of CRH-R1 mRNA variants. Negative controls containing water instead of first-strand cDNA were also used. Each sample was normalized on the basis of its 18S ribosomal RNA content (housekeeping gene). The 18S quantification was performed with a TaqMan ribosomal RNA reagent kit (Applied Biosystems) using the protocol of the manufacturer. All samples were run in triplicate, and results were calculated with reference to the amplification of 18S rRNA using comparative threshold cycle (C_T) method for relative quantitation (ABI Prism 7000 SDS version 1.1 software; Applied Biosystems).

The results were expressed as mean ± SE. Relative gene expression of target mRNA was normalized to a calibrator that was chosen to be the basal condition (untreated sample). Results were calculated with the $\Delta\Delta C_T$ method; they were expressed as the n-fold differences in gene expression relative to 18S rRNA and calibrator and were determined as follows: n-fold = $2^{-(\Delta CT\ sample\ -\ \Delta CT\ calibrator)}$, where the parameter C_T is defined as the fractional cycle number at which the PCR reporter signal passes a fixed threshold. ΔC_T values of the sample and calibrator were determined by subtracting the average C_T value of the transcript under investigation from the average C_T value of the 18S rRNA gene for each sample.

Qualitative RT-PCR analysis

The conditions for the conventional (qualitative) RT-PCR of CRH-R1 mRNA were as follows: 95 C for 15 sec, 64 C for 15 sec, and 72 C for 30 sec, in a total of 35 cycles with a final extension step at 72 C for 7 min. Primers sequence were as follows: forward, 5′-CAAACAATGGCTACCGGGAG-3′; and reverse, 5′ACACCCCAGCCAATGCAGA-3′. These primers amplified a 475-bp DNA fragment present in all CRH-R1 mRNA variants except CRH-R1β and a 538-bp DNA fragment specific for CRH-R1β. Ten microliters of the reaction mixture were subsequently electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide, using a 1-kb DNA ladder (Invitrogen) to estimate the band sizes. As a negative control for all of the reactions, distilled water was used in place of the cDNA. The PCR products were sequenced in an automated DNA sequencer (Department of Biological Sciences, The University of Warwick), and the sequence data were analyzed using Blast Nucleic Acid Database Searches from the National Center for Biotechnology Information.

Western blot

Cells were lysed in 150 μl Laemmli's buffer. The lysates were sonicated for 30 sec, and, after boiling for 5 min, proteins were spun down and 15 μl of the supernatant was loaded on 10%SDS-polyacrylamide gels. Gels were run at 200 V for approximately 1 h. Proteins were electrophoretically transferred to a polyvinylidene difluoride filter at 100 V for 1 h. The filters was initially incubated in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 5% milk (wt/vol) for 1 h at room temperature and, after a brief wash with TBS-T, were incubated with primary antisera. The primary antibodies for PTGS2 and CRH-R1/2 were used at a 1:1000 dilution in TBS-T overnight at 4 C (CRH-R1/2) or at room temperature for 1 h (PTGS2), whereas the IκBα antibody was used at a 1:500 dilution (overnight at 4 C). Equal protein loading was confirmed by using anti-GAPDH antibodies (1:40,000 dilution in TBS-T at room temperature for 1 h). The filters were washed thoroughly for 30 min with TBS-T, before incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000) for 1 h at room temperature. After an additional washing step for 30 min with TBS-T, antibody complexes were visualized using enhanced chemiluminescence. In some experiments, IκBα and GAPDH probed blots were incubated with goat antirabbit IRDye 800-conjugated IgG at 1:6000 in 10 ml of blocking buffer with gentle agitation for 1 h at room temperature. Finally, the blot was washed two more times each with 15 ml of TBS-T and once with TBS only. The membrane was dried, visualized, and analyzed on the Odyssey Infrared Imaging System (LI-COR Biosciences, Cambridge, UK).

Measurement of cAMP production

Myometrial cells were plated in 12-well plates, and, when they reached 70–80% confluency, they were transferred in DMEM for 12 h and treated with 1ng/ml IL-1β for 18 h. At the end of the incubation period, media was aspirated and cells were washed with plain DMEM. Then, stimulation buffer (DMEM containing 10 mM $MgCl_2$ and 1 mg/ml 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor) was added and cells were incubated for 20 min at 37 C. After washing with 1 ml DMEM, cells were stimulated with 100 nM CRH or 10 μM forskolin (to induce direct adenylyl cyclase activation) for 15 min. The reaction was stopped by adding 10 μl of concentrated HCl for 15–20 min at room temperature. Subsequently, cells were scraped and transferred in 1.5 ml tubes. After a brief spin, lysates were stored at −20 C. The cAMP levels were determined by a commercially available ELISA kit (low pH) from R & D Systems (Minneapolis, MN).

Confocal microscopy

Myocytes were grown on aminopropylethoxysilane-treated glass coverslips. To monitor NF-κB(p65) translocation, serum-deprived cells were treated with 1 ng/ml IL-1β for 30–60 min and fixed with 4% paraformaldehyde in PBS. After permeabilization with PBS containing 0.01% Triton X-100 and 3% BSA, cells were incubated with p65 antibody (1:100) overnight at 4 C. After three brief washes with PBS, slides were incubated for 1 h at room temperature with 1:400 antirabbit Alexa Fluor594. After three washes with PBS, the slides were mounted with Vectashield Hard Set TM mounting medium for fluorescence (Vector Laboratories, Burlingame, CA) or *Slowfade* gold antifade reagent with DAPI (Invitrogen). For detection of CRH-R, fixed cells were incubated with 1:100 CRH-R1/2 antibody overnight in the presence or absence of 1 μM blocking peptide. On the following day, the slides were washed and incubated with Alexa Fluor594 and Alexa Fluor488-phalloidin. The slides were mounted as described above. The slides were examined under an oil immersion objective (×63) using a Leica (Deerfield, IL) DMRE laser scanning confocal microscope with TCS SP2 scan head.

Data analysis and statistical methods

Results from triplicate experiments were pooled and analyzed by one-way ANOVA with Student-Newman-Keuls multiple comparison methods, using SigmaStat version 3.0 (SPSS, Chicago, IL).

Results

Determination of myometrial CRH-R1 mRNA variant expression

Previous studies (12) using qualitative RT-PCR analysis for CRH-R1 mRNA variants identified R1α, R1β, and R1c mRNA to be present in both preterm and term pregnant myometrium, whereas R1d mRNA was only present in term myometrium. Quantitative analysis of RNA isolated from human pregnant (term and preterm) myometrium with amplified CRH-R1 (total) mRNA-specific sequences showed a low number of C_T (30–32 cycles), indicative of substantial levels of CRH-$R1_T$ mRNA. CRH-R1β and R1c as well as R1d were also amplified from both term and preterm myometrium (reflecting probably the higher sensitivity and specificity of the quantitative RT-PCR method used); however, the C_T values of these variants mRNA amplification were higher (33–35 cycles), suggesting lower levels of expression (data not shown). Furthermore, comparison of the mRNA concentrations showed that the expression levels of the CRH-R1 mRNA variants relative to the total CRH-$R1_T$ mRNA levels was 4% for the R1β, 1% for the R1c, and 20% for the R1d. These data suggest that the majority of CRH-$R1_T$ mRNA amplification signal probably reflects CRH-R1α mRNA levels expressed in human myometrium (CRH-$R1_T$α). These ratios cannot be used for an accurate quantitation of each CRH-R1 mRNA levels but can rather provide a relative estimation of comparative receptor variant expression.

Studies on human preterm nonlaboring, preterm laboring, term nonlaboring, or term laboring human myometrium (n = 6 for each group) showed that the expression of both CRH-$R1_T$α and CRH-R1β mRNAs were significantly higher (by 2.5- and 0.5-fold, respectively) in term compared with preterm nonlaboring myometrium (Fig. 2A). In contrast, CRH-R1c and R1d mRNAs were not altered (Fig. 2A). Furthermore, active labor in both preterm and term groups was associated with a significant increase (by 3- and 2-fold, respectively) of CRH-$R1_T$α mRNA and CRH-R1d mRNA (by 2.5- and 3.5-fold, respectively). In contrast, CRH-R1β mRNA showed a significant decrease (by 40 and 70%, respectively) in laboring myometrium (both preterm and term) compared with nonlaboring myometrium, whereas no change was observed in CRH-R1c mRNA levels (Fig. 2B).

CRH-R mRNA and protein expression in primary myometrial smooth muscle cells

The differential expression levels of CRH-R1 mRNA transcripts in human myometrial biopsies during labor was further investigated in primary myometrial smooth muscle cells. Qualitative RT-PCR studies confirmed the expression of multiple CRH-R1 (including CRH-R1β) mRNAs (Fig. 3A). Moreover, indirect immunofluorescence with a CRH-R1/2-specific antibody was used to monitor distribution of CRH-R and counterstaining with phalloidin to detect filamentous actin (F-actin) stress fibers and the nuclear stain DAPI. Confocal microscopy studies demonstrated a strong CRH-R-positive immunostaining in the plasma membrane and the cytoplasm (Fig. 3B); interestingly, the plasma membrane signal was not evenly distributed in the cell membrane and was confined in specific membrane areas. Also, apparent colocalization between the CRH-R and F-actin immunostaining was observed in the plasma membrane (Fig. 3B, *yellow*), potentially suggestive of some common subcellular localization of CRH-R and actin stress fibers. No staining was detectable in cells in which the primary anti-CRH-R antibody was omitted or when cells were preincubated with a specific CRH-R antibody-blocking peptide (Fig. 3C).

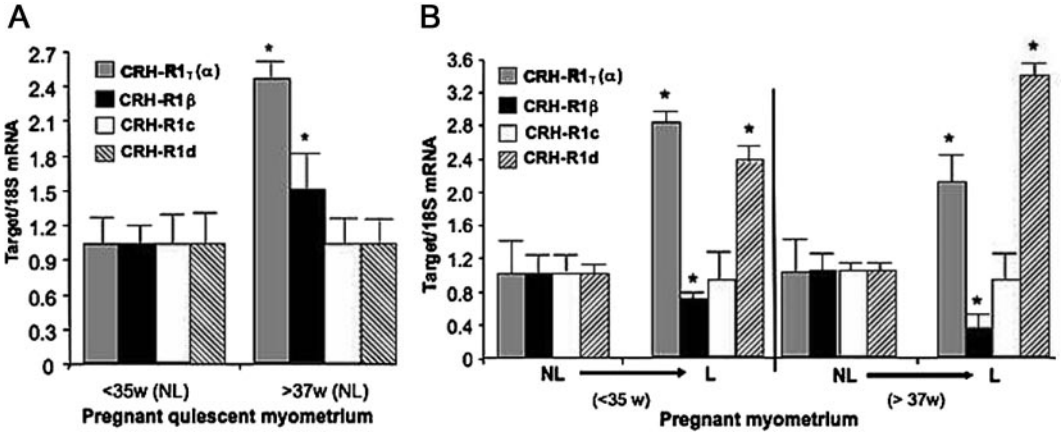

FIG. 2. Comparative studies of expression levels of CRH-R1 mRNA variants in gestational myometrial tissues (n = 6 for each group) in relation to progression toward term (A) or the onset of labor (preterm or term) (B). Relative expression was determined by real-time quantitative RT-PCR (TaqMan; Applied Biosystems). Amplification runs in triplicate were performed with cDNA preparations reversibly transcribed in the same reaction from 500 ng total RNA in each case. Results are expressed as mean values ± SEM of relative mRNA expression levels normalized against the levels of the housekeeping gene 18S RNA. *, $P < 0.05$ compared with preterm nonlaboring (A) or nonlaboring (B) myometrium values. The mean results of nonlaboring preterm myometrium (A) or nonlaboring preterm and term (B) were arbitrarily assigned the value 1. NL, Nonlaboring; L, laboring.

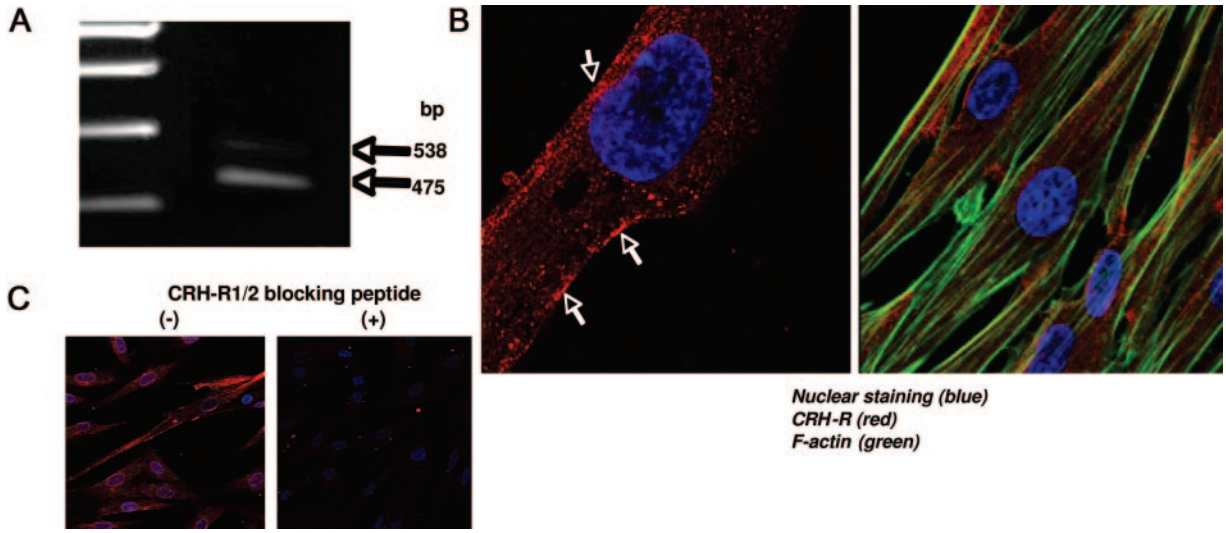

FIG. 3. A, RT-PCR amplification of CRH-R1 variants sequences from RNA extracted from primary human myometrial cell cultures. Specific primers able to amplify CRH-R1α, R1β, and R1d mRNA were used as described in *Materials and Methods*. Primer sets were designed to amplify a 475-bp fragment, present in CRH-R1α and R1d (but not R1c) mRNA, or a 538-bp fragment, present in CRH-R1β mRNA. PCR products were resolved on 1.2% agarose gel and stained with ethidium bromide. The identities of the fragments were confirmed by direct nucleotide sequencing. B and C, Visualization of CRH-R distribution in human pregnant myometrial cells by indirect immunofluorescence confocal microscopy using specific primary antibodies for CRH-R and Alexa Fluor594 secondary antibody (*red*) in the presence or absence of CRH-R blocking peptide (1 μM) as described in *Materials and Methods*. Distribution of F-actin and stress fibers was also monitored by double staining with Alexa Fluor488-phalloidin (*green*). Cell nuclei were stained with the DNA-specific dye DAPI (*blue*). Identical results were obtained from six independent experiments.

Effects of IL-1β on CRH-R1 and PTGS2 mRNA and protein expression in primary myometrial smooth muscle cells

It is well established that proinflammatory cytokines, especially IL-1β, are important regulators of myometrial smooth muscle cell gene expression during pregnancy (17). To confirm the functional activity of IL-1β in these cells, preliminary experiments were performed in cells treated with IL-1β for various time intervals, and expression levels of both PTGS2 mRNA and protein were determined by quantitative RT-PCR and immunoblot analysis using a specific antibody. Results (data not shown) showed that exposure of myometrial smooth muscle cells to IL-1β (1 ng/ml) significantly up-regulated PTGS2 mRNA and protein levels in a time-dependent manner; IL-1β significantly increased PTGS2 mRNA after 2 h of incubation and achieved maximal responses after 18 h (300- and 140-fold increase in mRNA and protein levels, respectively). Moreover, IL-1β treatment for 18 h induced myometrial cell CRH-R1$_T$α mRNA expression by 3-fold but significantly attenuated myometrial CRH-R1β mRNA expression by 40% (Fig. 4), without affecting CRH-R1c (data not shown) or R1d mRNA levels (Fig. 4). Furthermore, IL-1β treatments for 2 or 6 h had no effect on myometrial cell CRH-R1 (total), CRH-R1β, and CRH-R1d mRNA expression levels (Fig. 4). The effect of IL-1β was dose dependent, and a small but significant effect (0.8-fold increase above basal) on CRH-R1 mRNA was observed when IL-1β (0.1 ng/ml) was used (data not shown). Increasing concentrations of IL-1β up to 10 ng/ml did not exert any additional induction of CRH-R1 mRNA levels (data not shown).

Signaling pathways mediating IL-1β effects in myometrial cells

NF-κB is a major key intermediate of IL-1β actions in myometrial cells (15). Confocal microscopy studies using a specific p65 (RelA) antibody (Fig. 5A) demonstrated that, in untreated cells, p65 immunoreactivity was exclusively found in the cytoplasm. The potential role of NF-κB on IL-1β-induced PTGS2 and CRH-R1 mRNA variant expression was

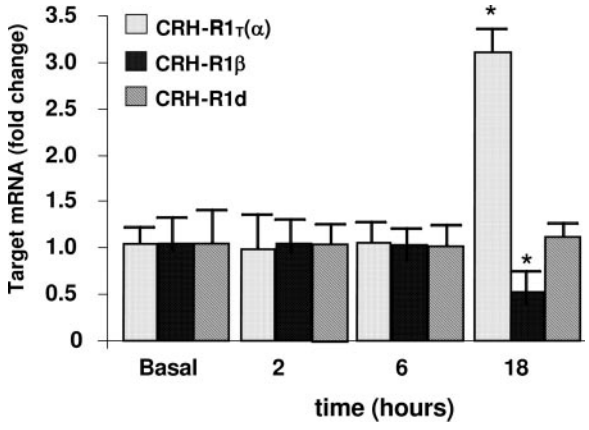

FIG. 4. Time course of the IL-1β effect on CRH-R1 (total), CRH-R1β, and CRH-R1d mRNA level in human myometrial smooth muscle cells. Cells isolated from myometrium of nonlaboring women (n = 6) were treated with IL-1β (1 ng/ml) for various time intervals, and target mRNA were determined by real-time quantitative RT-PCR (TaqMan; Applied Biosystems) as described in *Materials and Methods*. Data are expressed as mean values ± SEM of relative mRNA expression levels normalized against 18S RNA. *, $P < 0.05$ compared with basal (untreated). The mean results of basal untreated mRNA concentrations were arbitrarily assigned the value 1.

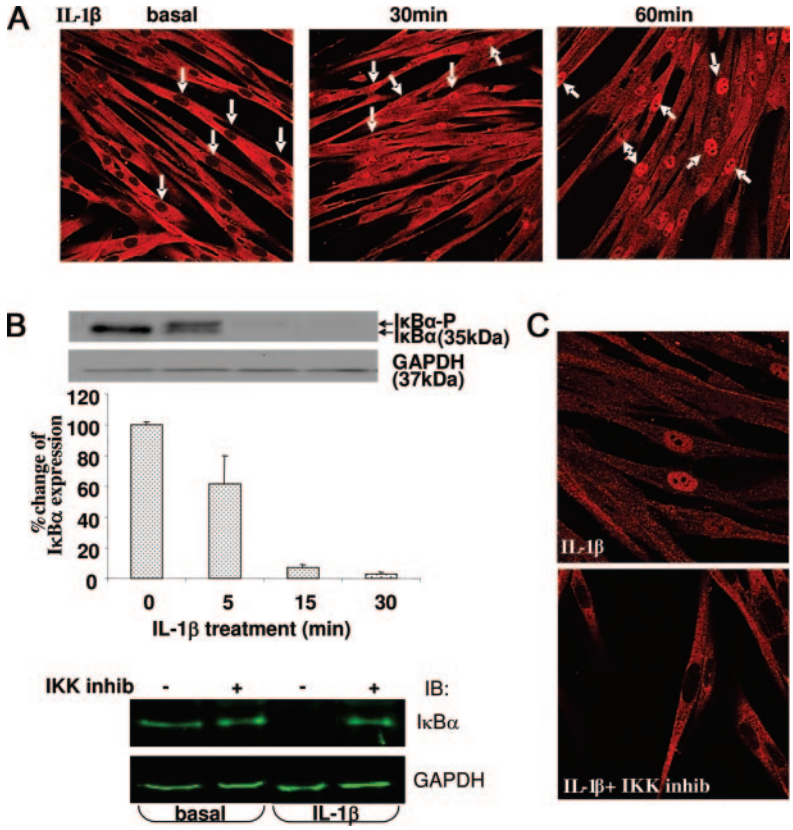

FIG. 5. IL-1β-induced activation of the NF-κB pathway. A, Human smooth muscle cells isolated from myometrium of nonlaboring women (n = 6) were treated with IL-1β (1 ng/ml) for various time intervals, and p65 (RelA) nuclear translocation was monitored by indirect immunofluorescence confocal microscopy using specific primary antibodies for p65 and Alexa Fluor594 secondary antibody (*red*) as described in *Materials and Methods*. In some experiments (C), cells were pretreated with or without 25 μM of the IKK inhibitor II Wedelolactone for 4 h, before IL-1β addition for 1 h. Identical results were obtained from four independent experiments and at least 20 cells were examined in each experiment. B, Representative Western blots of IκBα expression of cells stimulated with IL-1β (1 ng/ml) for various time intervals. After cell lysis and centrifugation, supernatants were subjected to SDS-PAGE and immunoblotted (IB) with antibodies for IκBα or GAPDH (protein loading control). After incubation with horseradish peroxidase-conjugated goat antirabbit antibody, complexes were visualized by enhanced chemiluminescence (*top*). Alternatively, immunoreactivity was detected by secondary antibodies conjugated to IRDye 800 (near-IR) and the Odyssey detection system as described in *Materials and Methods* (*bottom*). The data represent the mean ± SEM of three estimations from three independent experiments. In some experiments (*bottom*), cells were pretreated with or without 25 μM of the IKK inhibitor II Wedelolactone for 4 h, before IL-1β addition for 30 min.

investigated. Exposure of myometrial cells to IL-1β (1 ng/ml) induced nuclear translocation of p65 within 30 min, a response that was sustained for at least 1 h. Furthermore, IL-1β actions induced rapid phosphorylation (indicated by the appearance of another immunoreactive protein with a slightly higher molecular weight) and subsequent (almost complete) degradation of IκBα, the cytoplasmic protein that retains NF-κB in an inactive form through association and masking the nuclear localization sequence (15) within 30 min. The role of the upstream IKK was evaluated by the use of a specific IKK inhibitor II, Wedelolactone (18), and determination of myometrial IκBα degradation after IL-1β-treatment. Pretreatment of cells with 25 μM Wedelolactone for 4 h abolished IL-1β-induced IκBα degradation and p65 (RelA) nuclear translocation (Fig. 5, B and C). Most importantly, the IKK inhibitor significantly attenuated IL-1β-induced up-regulation of both PTGS2 and CRH-R1 mRNA expression (Fig. 6A).

Previous investigations have shown that IL-1β effects on myometrial PTGS2 up-regulation involve members of the family of MAPKs (19), namely ERK1/2 and p38 MAPK. Our results also showed that both of these kinases are phosphorylated and activated after acute exposure of myometrial cells to IL-1β (1 ng/ml) for 10 min (data not shown). The role of these kinases on IL-1β-induced regulation of myometrial target gene expression was investigated by using U0126 (MEK1/2 inhibitor) and SB203580 (a p38 MAPK inhibitor). Pretreatment of myometrial cells with each inhibitor significantly attenuated by 70–85% IL-1β effects on PTGS2 and CRH-R1 mRNA expression (Fig. 6B).

Effects of IL-1β on CRH-induced myometrial cAMP response

One of the major signaling pathways mediating CRH effects in human myometrial cells involves activation of the $G\alpha_s$-adenylyl cyclase pathway (20). The functional consequences of IL-1β-induced myometrial CRH-R1 up-regulation was investigated by pretreatment of cells with IL-1β for 18 h, followed by determination of intracellular cAMP production in response to CRH stimulation. Surprisingly, al-

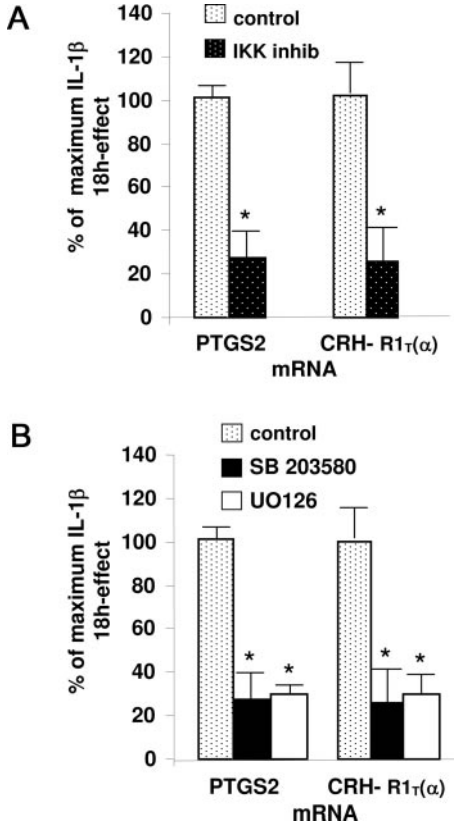

FIG. 6. Effect of IKK, ERK1/2, and p38 MAPK inhibitors on IL-1β-induced effects on myometrial PTGS2 and CRH-R1 gene expression. After pretreatment of cells with Wedelolactone (A) and U0126 and SB203580 (B) and stimulation with IL-1β (1 ng/ml) for 18 h, RNA was extracted, and PTGS2 and CRH-R1 mRNA levels were determined by real-time quantitative RT-PCR [TaqMan (Applied Biosystems) and Lightcycler (Roche Applied Science), respectively] as described in *Materials and Methods*. Data are expressed as mean ± SEM values of relative mRNA expression levels normalized against 18S or GAPDH RNA. *, $P < 0.05$ compared with inhibitor-untreated values.

though IL-β-treatment increased basal and forskolin-stimulated cAMP levels (by 10- and 2-fold, respectively), it significantly impaired CRH-induced cAMP production (by 85 ± 7%), suggesting that myometrial CRH-R1 mRNA and protein up-regulation was not associated with increased functional activity and cAMP production (Fig. 7).

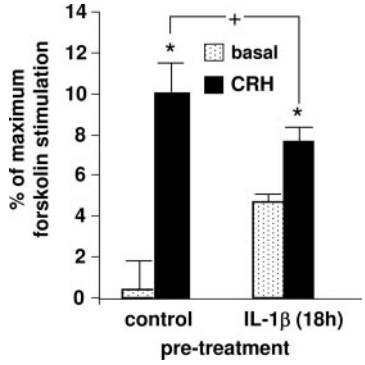

FIG. 7. Effect of IL-1β on CRH-induced cAMP production from human pregnant myometrial cells. Cells were pretreated with IL-1β (1 ng/ml) for 18 h, followed by incubation with 100 nM CRH for 10 min at 37 C. Given that IL-1β pretreatment affected both basal and forskolin-induced adenylyl cyclase activity, results were calculated as percentage of forskolin-induced cAMP release, which was considered as maximum (100%) for each group (treated or untreated). Results are representative of six separate cell culture preparations. Each *point* is the mean ± SEM of four estimates. *, $P < 0.05$ compared with basal; $^{+}$, $P < 0.05$ compared with CRH-stimulated values in cells without IL-1β pretreatment.

Discussion

Human pregnancy is associated with changes in the myometrial CRH-R variant expression profile and functional activity, consistent with the important role(s) of CRH and CRH-related peptides in the control of myometrial transition from a state of quiescence to one of increased contractility (3, 5, 12, 13, 21). These receptors appeared to contribute to maintenance of myometrial relaxation during pregnancy (7) through activation of the adenylyl cyclase/cAMP pathway (21). Our quantitative RT-PCR data clearly demonstrated that, as pregnancy progresses toward term, there is an increased transcription of the CRH-R1 gene in quiescent myometrial tissue. Most importantly, our results suggest that mechanisms activating myometrial contractility and labor, either term or preterm, induce increased transcription of the CRH-R1 gene and accelerate the (unknown) splicing mechanisms involved in the generation of downstream CRH-R1 mRNA splice variants. Analysis of the structural characteristics of myometrial CRH-R1 variants suggests that there are at least two distinct levels of splicing events (Fig. 1). Use of quantitative RT-PCR probes capable of distinguishing between different mRNA variants allowed us to investigate expression of individual CRH-R1 mRNA variants. The finding that in laboring myometrium there is decreased mRNA expression of the pro-CRH-R1 variant CRH-R1β with simultaneous increase of the "total" CRH-R1 mRNA (that reflects the mRNA levels of the fully active CRH-R1α) and the signaling-deficient variant CRH-R1d suggests induction of the two splicing events regulating individual expression of CRH-R1 variants by removing sequentially exons 6 and 13. These findings expand previous studies using a semiquantitative RT-PCR method that showed increased CRH-R1 mRNA expression in the lower myometrial segment, but not the fundus, in both preterm and term labor (22). Therefore, it appears that labor is associated with increased levels of active CRH-R1 receptors; this coupled with a decreased activity of the adenylyl cyclase/cAMP pathway might allow CRH to activate other signaling pathways and exert procontractile effects during the development of labor.

The mechanisms regulating CRH-R1 alternative splicing are essentially unknown. Studies in keratinocytes suggest that environmental stimuli such as UV irradiation as well as intracellular messengers such as cAMP and phorbol esters can induce CRH-R1 splicing (23). Furthermore, CRH-R heterogeneity has been shown previously in human myometrial membrane extracts (24), and the functional activity of some receptor variants appeared to be differentially regulated by oxytocin (9, 10). Although the lack of suitable antibodies able to distinguish between different CRH-R1 variants does not allow additional investigations at the level of individual protein expression and functional response, these results strongly suggest that the CRH-R1 gene transcriptional ac-

tivity and splicing is specifically targeted by the mechanisms involved in the onset of labor.

To investigate further the expression of CRH-R1, primary human myometrial cells were used, obtained from term human quiescent myometrial biopsies. This cellular system can provide useful information about the mechanisms regulating myometrial CRH-R expression and activity, although it cannot accurately reflect changes associated with the onset of labor because it is likely that cell-culturing conditions may alter cell phenotype. We were able for the first time to demonstrate uneven distribution of CRH-Rs in the plasma membrane. This finding strongly suggests "hotspots" of receptor expression, probably reflecting "confined" CRH-R signaling networks in specific areas. These experiments also showed significant cytoplasmic staining for CRH-R, potentially indicating newly synthesized unprocessed receptors, internalized mature CRH-R, or cytoplasmic forms of specific CRH-R variants. Moreover, our *in vitro* studies in myometrial cells identified IL-1β as a potential regulator of CRH-R1 gene transcription and splicing pattern. IL-1β, like many other proinflammatory cytokines, has been proposed to play important role in the onset of labor and the pathogenesis of infection-induced preterm labor (25). Our results suggest that IL-1β can potentially target CRH-R1 gene transcription and splicing mechanisms targeting exon 6, but not exon 13, of the CRH-R1 gene; these interactions appeared to involve two members of the MAPK family of proteins, ERK1/2 and p38 MAPK. In a variety of cells, these kinases exert their effects through phosphorylation of p65 and modulation of the NF-κB transcriptional activity (26). NF-κB is a critical component of pathways mediating IL-1β actions in human myometrium and other feto-maternal tissues (27) and increased p65 (RelA):p50 heterodimer DNA binding has been demonstrated in laboring myometrium and linked with increased transcriptional activation to specifically modulate changes in expression of genes critical for myometrial activation and contraction (28). Our results point toward an important role for NF-κB in IL-1β effects on CRH-R1 gene regulation and splicing, consistent with the presence of NF-κB recognition elements in the CRH-R1 promoter (14). In our experimental paradigm, IL-1β action induced phosphorylation of IκBα through an IKK-mediated mechanism that releases p65 (RelA) and allows its translocation to the nucleus (15). Other studies have showed that IL-1β actions on myometrial cells can modulate endothelin B receptor but not endothelin A receptor mRNA and protein (29); thus, CRH-R1 IL-1β interactions may be part of a much larger transcriptional modification in the myometrial cells.

Surprisingly, increased CRH-R1 gene transcription and generation of receptor splice variants was not associated with increased CRH signaling activity. On the contrary, prolonged exposure of cells to IL-1β significantly impaired CRH-induced cAMP production, although the IL-1β pretreatment significantly augmented basal adenylyl cyclase activity, in agreement with previous studies (30). It is likely that this is directly related to repression of the myometrial $G\alpha_s$ gene by NF-κB that has been reported recently (31). The possibility that IL-1β pretreatment might induce expression of signaling-deficient variants of CRH-R1 such as R1c and R1d was excluded by our real-time PCR experiments. Interestingly, the myometrial IL-1β/NF-κB and cAMP/protein kinase A (PKA) signaling pathways can interact at multiple levels; exposure of myometrial cells to IL-1β results in a significant up-regulation of cAMP-phosphodiesterase (PDE) 4, which is the predominant family of PDEs expressed in human myometrium (30) through a mechanism involving prostaglandin E_2 (PGE_2) production and subsequent cAMP augmentation. Also, myometrial p65 (RelA) appears to be specifically associated with PKAcα (as well as IκBα), through an interaction that potentially involves the phosphorylation of p65 at serine-536 (28). This association might contributes to p65 inactive state until the cell is exposed to a p65 inducing stimuli (*e.g.* IL1-β).

One of the main actions of IL-1β in human myometrium involves the up-regulation of PTGS2 enzyme and increased production of PG, such as PGE_2 (19). Our previous studies (20) have shown that short (0.5–2 h) as well as prolonged (8–18 h) treatments with CRH can attenuate myometrial PGE_2 release, and only the prolonged effect of CRH can be inhibited by coincubation with IL-1β. This coupled with the findings of the present study might suggest that IL-1β acts to diminish the CRH-induced cAMP response through modulation of CRH-R1 gene transcription and splicing, up-regulation of PDE4, and/or down-regulation of $G\alpha_s$, thus implicating the myometrial cAMP/PKA pathway in the inhibition of PGE_2 synthesis and release.

In conclusion, we provide conclusive evidence that the onset of labor (preterm or term) is associated with increased transcription of the myometrial CRH-R1 gene and altered splicing events that down-regulate the pro-CRH-R1 mRNA encoding the R1β variant but up-regulating receptor variants such as CRH-R1d, downstream of CRH-R1α. IL-1β appears to be one of the signals involved in the regulation of the CRH-R1 gene through a pathway involving activation of NF-κB, the classical mediator of IL-1β intracellular events, as well as at least two members of the MAPK family of proteins, ERK1/2 and p38 MAPK. Most importantly, IL-1β actions lead to dampening of the CRH-induced cAMP response, an event that might contribute to the procontractile intracellular environment required for the onset of labor. It is attractive to speculate that up-regulation of functional CRH-R1s might lead to activation of alternative signaling cascades that might help in the transition of the uterus from a state of quiescence to one of increased contractility and active labor.

Acknowledgments

We thank Drs. Mark Johnson and Dev Sooranna for useful discussions.

Received January 22, 2007. Accepted April 2, 2007.

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This work was supported by a Wellcome Trust University Award (to D.K.G.) and a Warwick Private Hospitals Charitable Trust grant (to M.V.).

Disclosure Statement: The authors have nothing to disclose.

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***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**