Expression of corticotrophin-releasing hormone receptor mRNA and protein in the human myometrium

B Rodríguez-Liñares, E A Linton and **S Phaneuf**

Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Women's Centre, John Radcliffe Hospital, Oxford OX3 9DU, UK
(Requests for offprints should be addressed to B Rodríguez-Liñares)

Abstract

The reported effects of corticotrophin-releasing hormone (CRH) on human myometrium support the existence of specific receptors for the hormone in this tissue. We have used the reverse transcriptase–polymerase chain reaction (RT–PCR) technique to study the expression of mRNA coding for the CRH R1 and R2 receptors. RT–PCR of total RNA from both nonpregnant and pregnant myometrium using specific primers resulted in amplification products of the expected sizes for the R1α and R2α CRH receptors. The identity of these amplification products was confirmed by specific restriction digests and sequencing. Immunohistochemistry using a rabbit antibody raised against a specific domain of the CRH R1 receptor demonstrated that the R1 mRNA is translated into protein and confirmed that it is the uterine smooth muscle cells from both nonpregnant and pregnant women that bear this receptor. Our results suggest that CRH may play a role in human pregnancy at the myometrium.
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Introduction

Although corticotrophin-releasing hormone (CRH) was initially identified as a hypothalamic hormone controlling the stress response, the widespread distribution of the peptide and its binding sites in the brain and periphery has led to the recognition that it also has effects on the endocrine, cardiovascular, gastrointestinal and immune systems (for review, see Owens & Nemeroff 1991). CRH exerts its actions by binding to specific cell surface receptors on its target tissues. At present, two different subtypes of CRH receptor, R1 and R2, have been cloned from human tissue and they belong to the calcitonin/vasoactive intestinal peptide/growth hormone-releasing hormone super-family of the seven transmembrane domain, G protein-coupled receptors (Chen *et al.* 1993, Vita *et al.* 1993, Liaw *et al.* 1996). The R1 receptor shows a high degree of interspecies homology and exists in two splice forms, α and β which occur in both human and rodent brain (Chang *et al.* 1993, Chen *et al.* 1993). The R2 receptor also has two splice forms (Lovenberg *et al.* 1995*a*), with the α variant described only in human and rat brain and the β form expressed in rodent peripheral tissues (Lovenberg *et al.* 1995*b*) but not so far identified in humans (Liaw *et al.* 1996). The R1 type presents greater selectivity for CRH whilst the R2 receptor exhibits preferential selectivity for the CRH-like peptides, sauvagine, urotensin and its mammalian counterpart, urocortin (Vaughan *et al.* 1995). The difference in the pharmacological profile and distribution of R1 and R2 receptors and their splice variants suggest that they may have different functions.

The placenta is an important source of circulating CRH (for review, see Perkins & Linton 1995*a*). The outer syncytiotrophoblast layer of placental chorionic villi produces CRH and releases it into the maternal circulation (Perkins & Linton 1995*b*). Maternal plasma levels of CRH rise with increasing gestational age, then rapidly fall to low basal values immediately following delivery. In pre-eclampsia and preterm labour, CRH levels rise earlier and to a greater extent than in normal pregnancy (Wolfe *et al.* 1988, McLean *et al.* 1995), whilst women who deliver post-term display lower plasma CRH levels (McLean *et al.* 1995). These observations suggest that this hormone plays an important role in the maintenance of pregnancy and in the onset of parturition. In search of such a role, several groups have focussed their attention on the myometrium as a target for CRH effects. Ligand binding studies from Hillhouse *et al.* (1993) have provided evidence for the presence of CRH binding sites that increase in affinity during pregnancy. Later studies demonstrated that this myometrial CRH receptor is G protein-linked to adenylate cyclase and presents multiple isoforms in both nonpregnant and pregnant women (Grammatopoulos *et al.* 1994, 1995). To date there is no indication of the identity of the myometrial CRH receptor(s). In the present study, we have examined the expression of CRH R1 and R2 receptors in human myometrial tissue by the reverse

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transcriptase–polymerase chain reaction (RT–PCR) and the localization of the CRH R1 receptor protein in myometrial tissue by immunohistochemistry.

Materials and Methods

Tissue collection

Pregnant term (38–39 weeks) human myometrium from women aged 36·7 ± 2·7 (n=6) was taken from the upper border of the uterine incision during elective lower-segment elective caesarean sections performed for previous caesarean section or cephalopelvic disproportion. Samples of myometrium from nonpregnant, premenopausal women aged 40·3 ± 2·5 (n=6) were obtained at hysterectomy performed for benign gynaecological disorders such as menorrhagia or dysmenorrhea. The uteri were excised longitudinally and samples were taken from the same region as in the pregnant tissue, about 5 mm from the endometrial and serosal surfaces. This investigation had the approval of the Central Oxfordshire Research Ethics Committee, and all patients gave informed consent. Rat cortex and heart used for control studies were dissected rapidly as described previously (Castro *et al.* 1996). All tissues were collected into liquid nitrogen and stored at − 80 °C until use.

RNA isolation and PCR amplification

Total RNA was prepared from tissue specimens by the guanidinium-isothiocyanate/caesium chloride method (Sambrook *et al.* 1989). cDNA was synthesised from RNA samples (2 μg) in 20 μl reaction mixture containing 100 units Moloney murine leukaemia virus reverse transcriptase according to the technical notes provided by the supplier (R&D System Europe, Abingdon, Oxon, UK) and using an oligo dT primer. The primer was annealed to RNA from the tissue of interest at 70 °C for 10 min, and the synthesis was carried out at 37 °C for 60 min. The PCR was carried out at 94 °C for 5 min, then 30 cycles at 94 °C for 1 min, 60 or 65 °C (depending on the set of primers used) for 1 min and 72 °C for 2 min, with a final elongation step at 72 °C for 5 min. The efficiency of all human RT products was tested by performing a PCR with primers (Chambers & Harris 1993) designed to amplify a 572 bp fragment of human glucocerebrosidase (Sorge *et al.* 1985). The CRH R1 receptor has been shown to exist in two different isoforms, α and β, the latter being an alternatively spliced form in which 29 amino acids are inserted into the first intracellular loop. The primers in this study were designed using regions shared by both variants and by both the human and rat sequences, so that they would recognise both mRNAs if present in rat and human tissues. The following primers (synthesised by R&D System Europe) for the CRH R1 receptor gene were used: forward R1FOR 275 5′ACAAACAATGGC TACCGGGA3′ 294 and reverse R1REV 866 5′TCATG GGGCCCTGGTAGAT3′ 848 generating fragments of 592 bp (for the α isoform) and 679 bp (for the β isoform). Numbering is based on the human CRH R1 sequence (Chen *et al.* 1993; Genbank accession number L23332). In rat and mouse, the R2 receptor also presents two splice forms, α and β, and only the former has been identified in humans so far. The β form is 431 amino acids in length and differs from the α form in the N-terminal region where the first 34 amino acids are replaced by 54 different amino acids. Primers were designed from a region shared by two splice forms common to rodents and the α human variant, so that they would recognise, but would not distinguish between, both α and β isoforms if present. Forward primer R2FOR 609 5′TGTGGAAGGCTGC TACCTG3′ 627 and reverse R2REV 1223 5′GTCTGC TTGATGCTGTGGAA3′ 1204 were synthesised to generate a fragment of 615 bp for the CRH R2 receptor gene. Primers that amplify a fragment of 174 bp of the human R2α sequence were R2αFOR 62 5′AGCTGCTCTTGG ACGGCT3′ 79 and R2αREV 235 5′CATTCCGGG TCGTGTTGTAC3′ 216. Numbering is based on the human CRH R2 sequence (Liaw *et al.* 1996; Genbank accession number U34587). The PCR fragments were resolved on 1·5 or 2% agarose gels and visualised under UV light by ethidium bromide staining. All primer sets were shown not to amplify a product from genomic DNA.

Enzyme digestion and sequencing

PCR fragments were isolated from agarose gels with the QIAquick gel extraction kit or purified from the reaction mixture with the QIAquick PCR purification kit (Qiagen Ltd, Dorking, Surrey, UK). The specific fragments generated by each PCR were verified by restriction enzyme digestion with BsiHKAI (New England Biolabs UK Ltd, Hitchin, Herts, UK) at 60 °C for 1 h.

Direct sequencing of PCR products obtained with the R1FOR/R1REV, R2FOR/R2REV and R2αFOR/R2αREV primer sets was carried out by a modified dideoxy chain termination method described by Roberts *et al.* (1991) and the thermosequenase cycle sequencing method (Amersham International, Amersham, Bucks, UK). Samples were separated in 8·3 M urea–6% polyacrylamide gel and visualised by autoradiography.

Immunohistochemistry

Six micron sections of frozen tissue were cut on a Leitz cryostat and allowed to dry in air overnight at room temperature. Samples were then rinsed with PBS prior to fixation by sequential 5 min washes in 80, 90, 100% 1:3 acetone/methanol. After rinsing with Tris buffered saline–Triton (TBT) (0·1 M Tris–HCl, 0·15 M NaCl, 0·25% BSA, 0·5% Triton X-100, pH 7·4) tissue sections were

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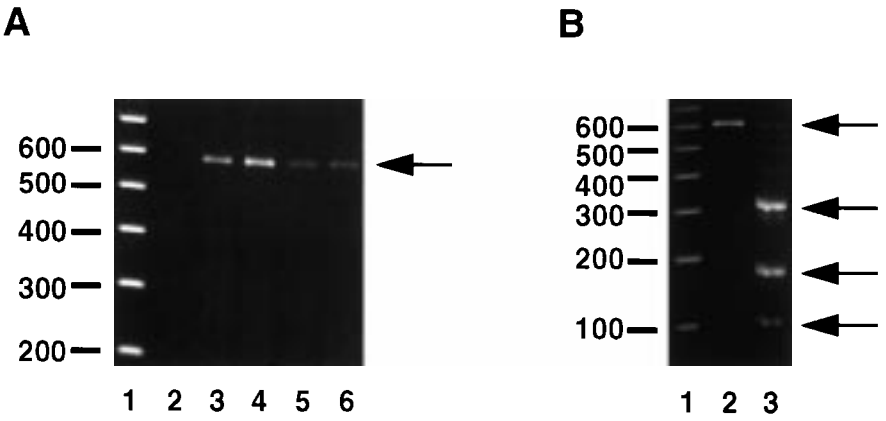

Figure 1 Expression of the CRH R1α receptor gene in human myometrium using R1FOR and R1REV primers. (A) PCR product of 592 bp from both nonpregnant (lanes 3 and 4) and pregnant women (lanes 5 and 6). The same result was obtained in four other patients in each group. Lane 1 contains the DNA marker. Lane 2 shows a PCR sample without RNA transcribed product (blank). (B) The 592 bp fragment from a nonpregnant patient (lane 2) was digested with the restriction enzyme BsiHKAI resulting in three products of 106, 174 and 312 bp (lane 3). The same result was obtained with a PCR fragment from a pregnant woman (not shown). Arrows denote PCR fragments.

blocked with 50% normal human serum (NHS) in TBT for 10 min. The rabbit polyclonal antiserum, coded JR2A, was raised in this laboratory against a non-transmembrane peptide sequence of the CRH R1 receptor (amino acid sequence 335–346) which bears no similarity to other members of this receptor subfamily. This antibody has previously been shown to detect specifically the CRH R1 receptor expressed in transiently transfected COS-7 cells, in AtT20 cells and in various tissue extracts including the cerebellum, cortex and pituitary gland (Castro *et al.* 1996). JR2A was added at 1:100 dilution in 10% NHS in TBT for 30 min. After rinsing with TBT, the following antibodies were used: 1:50 dilution of mouse anti-rabbit immunoglobulin, 1:50 dilution of rabbit anti-mouse immunoglobulin and 1:100 dilution of mouse immunoglobulin coupled to alkaline phosphatase (APAAP). All dilutions were prepared in TBT and the incubations allowed to proceed for 30 min prior to washing for 2 min in TBT. The alkaline phosphatase complex was detected as described previously (Perkins & Linton 1995*b*). Controls for immunostaining included the use of pre-immune rabbit serum and JR2A antibody preabsorbed overnight at 4 °C with the CRH R1 peptide fragment (JR2) used for antibody production.

Results

Expression of CRH R1α receptor gene in human myometrium

PCR amplification using primers R1FOR and R1REV designed to amplify a 592 bp fragment of the human and rat CRH R1α receptor, or a 679 bp fragment of the R1β receptor, resulted in only a 592 bp band when assayed on myometrial tissue from either nonpregnant or pregnant women as shown in Fig. 1A. Digestion of the PCR product with BsiHKAI generated the predicted bands of 106, 174 and 312 bp (Fig. 1B). Amplification of cDNA prepared from rat frontal cortex, where the CRH R1α has been reported to be present (Potter *et al.* 1994), resulted in the same 592 bp product. The restriction enzyme assay confirmed the identity of this PCR product (data not shown). The same set of primers did not produce a band when assayed on rat heart where only the CRH R2 receptor has been described (Lovenberg *et al.* 1995*a*) (data not shown).

Further evidence for the presence and identity of the CRH R1α receptor in this tissue was provided by the use of another forward primer (86–103) upstream of the CRH R1 receptor sequence than the R1FOR. Amplification of the same myometrial samples resulted in the predicted 781 bp product that was also digested by BsiHKAI as expected. When this product was used in a second PCR utilising the R1FOR and R1REV primers, the anticipated band of 592 bp was amplified (data not shown).

Finally, sequencing of the 592 bp fragment of CRH R1α receptor showed 100% homology with the reported sequence of the CRH R1 receptor gene (Chen *et al.* 1993) (data not shown).

Expression of CRH R2α receptor gene in human myometrium

Rat heart was initially used as a positive control (Lovenberg *et al.* 1995*a*) to show the expression of the

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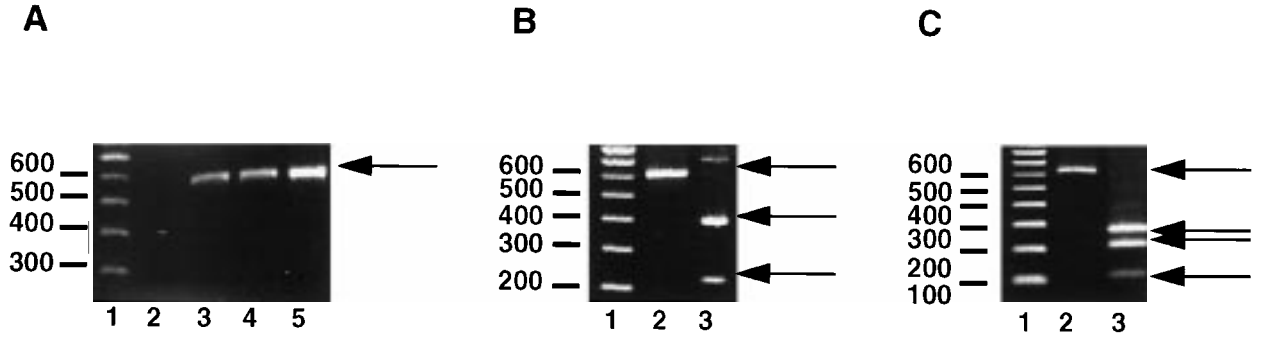

Figure 2 Expression of the CRH R2 receptor gene in rat heart and human myometrium using R2FOR and R2REV primers. (A) 615 bp PCR product from rat heart (lane 3), nonpregnant (lane 4) and pregnant myometrium (lane 5). Results are representative of four other patients. Lane 2 contains a PCR sample without RNA transcribed product. (B) The 615 bp band from rat heart (lane 2) was digested with BsiHKAI generating two products of 219 and 396 bp (lane 3). (C) The 615 bp product from nonpregnant myometrium (lane 2) was digested with BsiHKAI giving three products of 118, 218 and 279 bp (lane 3). The same result was obtained with PCR product from pregnant myometrium (not shown). In A, B and C, DNA markers are shown in lane 1. Arrows denote PCR fragments.

CRH R2 receptor gene with the primers R2FOR and R2REV. Primers were designed to amplify a 615 bp fragment of the CRH R2α and β receptor gene. When assayed on rat heart mRNA, these primers gave a product of the predicted size (Fig. 2A, lane 3). The RT–PCR assay on nonpregnant and pregnant myometrium using the R2FOR and R2REV primers also resulted in the same 615 bp product as shown in Fig. 2A (lanes 4 and 5).

The identity of these bands was confirmed by restriction endonuclease digestion using BsiHKAI. Due to differences between this particular region of the rat and human sequences of the CRH R2 receptor, this enzyme should cut the cDNAs differently depending on the species used. BsiHKAI would cut the rat fragment at one site, generating two products of 219 and 396 bp, whilst the human fragment would be cleaved at two sites, producing three products of 118, 218 and 279 bp. BsiHKAI would cut the equivalent 615 bp region of the CRH R1 receptor only once, providing additional support for the specificity of the CRH R2 primers. Figs 2B and C show that the digestion assay on both rat and human 615 bp fragments did indeed give the predicted products.

To determine whether the α isoform of the CRH R2 receptor is present in the human myometrium, primers R2αFOR and R2αREV were designed to amplify a 174 bp fragment of the human CRH R2α receptor. When used on human nonpregnant and pregnant myometrial mRNA, these primers resulted in the production of the predicted 174 bp fragment in both cases as shown in Fig. 3.

Finally, sequencing of the isolated 615 and 174 bp PCR products showed full homology with the reported sequence of the human CRH R2α receptor gene (Liaw *et al.* 1996) (data not shown).

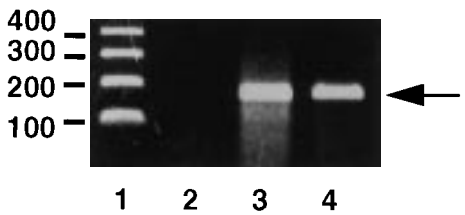

Figure 3 Expression of the CRH R2α receptor gene in human myometrium using R2αFOR and R2αREV primers, showing PCR product of 174 bp from both nonpregnant (lane 3) and pregnant myometrium (lane 4). Lane 1 contains the DNA marker. A sample from a PCR amplification carried out following a reverse transcriptase reaction containing no RNA was applied to lane 2. Results are representative of three patients. Arrow denotes PCR fragments.

Expression of CRH R1 receptor protein in human myocytes

To assess whether CRH receptor mRNA is translated in the human myometrium, immunohistochemical analysis was performed. First, rat frontal cortex, where the CRH R1 receptor has been reported to be present (Potter *et al.* 1994), was used as a positive control (data not shown). Immunoreactive CRH R1 protein was detected in the uterine smooth muscle cells in tissue from both nonpregnant and pregnant women (Fig. 4, panels A and C respectively). Staining was widespread though heterogeneous; some strongly positive smooth muscle cells were occasionally surrounded by others which were only weakly positive for the R1 receptor protein. This staining pattern occurred in both pregnant and nonpregnant myometrium. The pre-immune rabbit serum negative control is shown in panels B and D. No positive staining was seen in tissue immunostained with JR2A antibody which had been preabsorbed with the synthetic peptide JR2 (data not shown).

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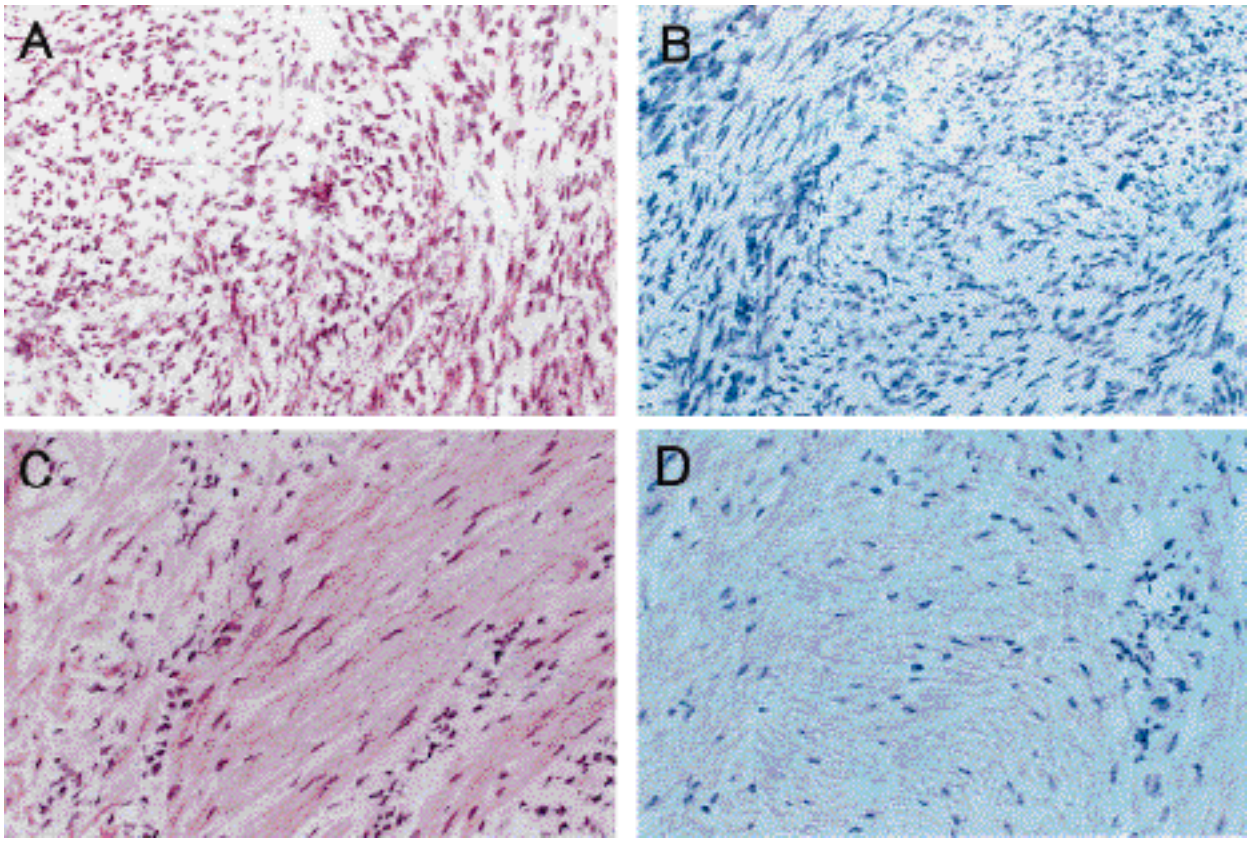

Figure 4 Immunolocalization of the CRH R1 receptor in cryosections of (A) human nonpregnant and (C) pregnant myometrium. Negative control-immune rabbit serum in these tissues are shown in B and D for nonpregnant and pregnant myometrium respectively. × 200 magnification. Results are representative of two other patients.

Discussion

There has been an interest in stress axis hormones and the initiation of labour since Liggins (1968) demonstrated the importance of ACTH and cortisol in parturition in sheep. Subsequent work from Wintour *et al.* (1986) showed that chronic infusion of CRH can also result in the early delivery of the lamb. CRH exerts numerous effects on pregnancy tissues which support a role for this hormone in the initiation of labour in humans. Normal parturition is associated with increased intrauterine prostaglandin (PG) release and CRH is known to upregulate placental and fetal membrane production of $PGF_{2\alpha}$ and PGE_2 (Jones & Challis 1989). At the myometrium, the most direct target for the peptide with respect to parturition, CRH itself has no intrinsic contractile activity, but it has been reported to enhance the contractile effect of $PGF_{2\alpha}$ (Benedetto *et al.* 1994). Quartero & Fry (1989) first described synergism between oxytocin and CRH in stimulating myometrial contractility, although more recent work has been unable to confirm this (Benedetto *et al.* 1994). Hillhouse's group have demonstrated that myometrial CRH binding sites do indeed exist, and they proposed that CRH may be involved in maintaining myometrial quiescence in pregnancy through stimulation of cAMP production, with the enhancement of myometrial contractility only coming into play at the beginning of labour following the uncoupling of adenylate cyclase (Hillhouse *et al.* 1993, Grammatopoulos *et al.* 1994, 1995). Their iso-electric focussing studies suggest that five isoforms of the CRH receptor exist, but it is not known whether any of these represent any of the R1 or R2 CRH receptor subtypes characterised to date.

The data presented here clearly demonstrate for the first time that the CRH R1α and R2α receptor mRNAs are expressed in the human myometrium from both pregnant and nonpregnant women. The possibility that a similar, yet distinct, transcription product for each of these was amplified is highly unlikely, as indicated by restriction endonuclease digestion, nested PCR and sequencing of PCR products. To our knowledge this is also the first description of the expression of the CRH R2α receptor gene in a human peripheral tissue, the location of this subtype previously thought to be limited to brain only.

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However, our experimental approach does not exclude the additional presence of the β isoform of the CRH R2 receptor in human myometrium. Primers designed to amplify a 270 bp fragment of the rat CRH R2β receptor gave the predicted band when assayed on rat heart mRNA, but did not produce a band with myometrial samples (results not shown). This result does not rule out the possibility that the CRH R2β receptor is present in human myometrium since the human sequence of this receptor subtype is only partially defined (Genbank accession number Y10153, Y10151). The human CRH R2β receptor sequence may differ from that of the rat and therefore not be recognised by the primers used in this study.

Many studies have demonstrated that CRH can exert actions by binding to several different cells, including immune (lymphocytes, macrophages) (McGillis *et al.* 1989, Webster *et al.* 1990) and vascular (endothelium, vascular smooth muscle) (Lei *et al.* 1993, Barker & Corder 1995) cells which are likely to be present in myometrial tissue in addition to the predominant cell-type, the myocyte. Here, we have used a previously characterised antibody (Castro *et al.* 1996) specific for the CRH R1 receptor to demonstrate that the R1 mRNA expressed in human myometrium is translated into protein and that this protein is indeed located on uterine smooth muscle cells within the tissue. Parallel studies for the R2 receptor protein were not possible due to the current lack of an available antibody specific for the human R2 receptor sequence; our attempts to produce such an antibody using the same approach as we used for the R1 receptor have so far resulted in low-titre antiserum insufficient for immunohistochemical work, probably as a result of the low immunogenicity of the limited number of specific amino acid sequences present in this receptor subtype. Whether the R2 receptor subtype protein is expressed in myometrial tissue in detectable quantity, and if so, in which cell-type is as yet unknown.

Myocytes from both nonpregnant and term pregnant myometrium were found to bear the R1 receptor. The heterogeneous immunocytochemical staining pattern observed here resembles that for the oxytocin receptor (OTR), which was also found in the myocytes in both pregnant and nonpregnant myometrium (Kimura *et al.* 1996), although CRH R1 receptor staining appears to be more extensive than OTR staining, especially in nonpregnant myocytes. The function of the CRH R1 receptor in nonpregnant myometrium is not known, but its presence here suggests that CRH may have other roles in addition to the control of myometrial activity in pregnancy. In support of a role for CRH in nonpregnant women, it has been shown that this hormone is present in nonpregnant myometrium (Clifton *et al.* 1996), ovaries (Mastorakos *et al.* 1994) and endometrium (Mastorakos *et al.* 1996) where it may participate in the inflammatory processes of ovulation and uterine physiology.

In conclusion, we have shown for the first time that CRH R1α and R2α receptor mRNA are expressed in human myometrium from both pregnant and nonpregnant women. CRH R1 receptor protein is definitively expressed in uterine myocytes. Further experiments are required to determine the cellular localization of the R2 receptor and it is possible that cultures of pure populations of myocytes may offer an alternative approach to the problem. Nevertheless, our results suggest a role for CRH in human pregnancy at a myometrial site.

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