Levels of corticotrophin-releasing hormone receptor subtype 1 mRNA in pregnancy and during labour in human myometrium measured by quantitative competitive PCR

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ABSTRACT

It is suggested that corticotrophin-releasing hormone (CRH) is involved in parturition. We have previously reported the presence of the CRH receptor subtype 1 (CRH R1) in human uterine myocytes. The aim of the present study was to investigate whether expression of the CRH R1 in myometrial tissue changes in pregnancy and labour. We used a quantitative competitive PCR method to measure the mRNA levels of this receptor in non-pregnant and in term pregnant myometrium before and at different stages of labour. The levels of mRNA for the housekeeping gene for glucocerebrosidase (GCB) were also determined. The results were expressed as a ratio of CRH R1 and

GCB mRNA levels. We have found that in pregnancy the CRH R1 is down-regulated from a ratio of 0.093 ± 0.011 in non-pregnant myometrium to 0.012 ± 0.005 ($P < 0.001$) in term non-labouring myometrium. No significant changes were observed in the CRH R1:GCB ratio in tissues sampled within 13 h (0.013 ± 0.004) from the start of labour. In summary, normalised levels of CRH R1 are down-regulated in pregnancy and do not change during labour. We speculate that our results do not support a direct role for the CRH R1 receptor in myometrial stimulation.

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INTRODUCTION

The placenta and fetal membranes are an important source of corticotrophin-releasing hormone (CRH) during pregnancy (Riley *et al.* 1991). Maternal plasma levels of CRH rise with increasing gestational age, peak at term and then rapidly fall to low basal values immediately following delivery. Circulating CRH-binding protein concentrations decrease at the end of pregnancy (Linton *et al.* 1993) resulting in a net increase in free and biologically active CRH, being highest at term (Campbell *et al.* 1987). In pregnancies complicated by pre-eclampsia and preterm labour, CRH levels rise earlier and to a greater extent than in normal pregnancy and CRH-binding protein levels are lower (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 a role for CRH in pregnancy and in the onset of

parturition. The myometrium is a potential site of action of circulating CRH. Work by Hillhouse's group has provided functional and pharmacological evidence for myometrial CRH-binding sites (Hillhouse *et al.* 1993, Grammatopoulos *et al.* 1994). Using reverse transcriptase–polymerase chain reaction (RT-PCR) we have recently subtyped the CRH receptors in human myometrium. We found that CRH R1 α and R2 α genes are expressed in human myometrium from both pregnant and non-pregnant women and immunohistochemical studies show that the R1 protein is expressed in uterine myocytes (Rodríguez-Liñares *et al.* 1998). Myocytes in culture express the CRH R1 but not the CRH R2 (J Woodman, B Rodríguez-Liñares, S Phaneuf & E A Linton, manuscript in preparation) suggesting that the CRH R2 identified in myometrial tissue originated from vascular smooth muscle or endothelium where this receptor subtype has recently been found (Jain *et al.*

1998). It has been proposed that CRH itself does not have an effect on contraction but potentiates oxytocin-induced (Quartero & Fry 1989) and prostaglandin (PG) $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced myometrial contractility (Benedetto *et al.* 1994, Petraglia *et al.* 1995). However, recent work by Simpkin *et al.* (1997) using spontaneously contracting myometrium failed to show any effect of CRH on myometrium from women at term before the onset of labour, either on spontaneous, $PGF_{2\alpha}$ - or oxytocin-induced myometrial contractility. CRH could also act indirectly on contractility through stimulation of PG synthesis in the decidua (Petraglia *et al.* 1995), fetal membranes and placenta (Jones & Challis 1989). We hypothesised that if CRH R1 is involved in myometrial contractility, its expression at this site should be modulated during pregnancy and labour.

In the present study, we have constructed homologous competitor fragments for the CRH R1 and glucocerebrosidase (GCB) mRNAs in order to investigate whether CRH R1 myometrial levels change in pregnancy and during labour. Using a quantitative competitive polymerase chain reaction (QC-PCR), we monitored the mRNA levels of this receptor and the housekeeping gene GCB in non-pregnant and term pregnant myometrium before the onset of labour and during different stages of labour.

MATERIALS AND METHODS

Tissue collection

Samples of myometrium from non-pregnant, premenopausal women (age 39 ± 1.7 years, mean $\pm$ S.E.M.; $n=5$) were obtained at hysterectomy performed for benign gynaecological disorders such as menorrhagia or dysmenorrhoea. The uteri were excised longitudinally and samples were taken from the same region as on the pregnant tissue, about 5 mm from the endometrial and serosal surfaces. Samples of pregnant myometrium were obtained at term (38–39 weeks gestation) from patients having a caesarean section either electively, before the onset of labour (indicated for previous sections or cephalopelvic disproportion; age 28.9 ± 2.1 ; $n=7$), or as an emergency following the spontaneous onset of labour (fetal distress or failure to progress; age 31.0 ± 1.0 ; $n=8$). The samples were obtained from the upper lip of the lower segment uterine incision. Histological analysis indicated that the lower segment samples consisted predominantly of myometrial smooth muscle cells. All patients were of mixed parity (0–3) and were delivered of healthy singleton infants. This investigation had the

approval of the Central Oxfordshire Research Ethics Committee, and all patients gave informed consent. All tissues were collected into liquid nitrogen and stored at -80°C until use.

Cell culture

AtT20/D16-16 mouse pituitary tumour cells, used as a positive control for the presence of the CRH R1, were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, 1 mM sodium pyruvate, 1% (v/v) minimum essential medium non-essential amino acids, 100 IU penicillin/ml and 100 μg streptomycin/ml. The cell line was maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were grown to confluence in 75 cm^2 plastic culture flasks and medium removed prior to RNA extraction of cells.

RNA isolation and RT-PCR amplification

Total RNA was prepared from tissue specimens and AtT20 cells by the guanidinium isothiocyanate/caesium chloride method (Sambrook *et al.* 1989). cDNA was synthesised from RNA samples (2 μg) in 20 μl of reaction mixture containing 100 units of Moloney murine leukaemia virus reverse transcriptase (RT) (R&D Systems Europe, Abingdon, Oxon, UK) and using an oligodT 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 reaction was carried out at 94°C for 5 min, then 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, with a final elongation step at 72°C for 5 min.

The following primers (synthesised by R&D Systems Europe) for the CRH R1 gene were used: CRH R1 forward (bases 275 to 294) and reverse (bases 866 to 848) generating a fragment of 592 bp (Rodríguez-Liñares *et al.* 1998). A schematic representation is shown in Fig. 1A.

Forward (bases 1182 to 1201) and reverse (bases 1753 to 1734) primers were synthesised to amplify a 572 bp fragment of the human GCB gene (Chambers & Harris 1993).

The PCR fragments were resolved on 1.5% agarose gels and visualised under UV light by ethidium bromide staining. All primer sets were shown not to amplify a product from genomic DNA.

Homologous competitive PCR

The homologous competitive PCR assay was based on the method of Celi *et al.* (1993). A 518 bp

FIGURE 1. Schematic representation of the production of 592 bp CRH R1 receptor fragment (A) and the 518 bp internal quantitative DNA standard (B).

internal cDNA standard or competitor was generated from cDNA by PCR using the following primers: (1) the 20-mer CRH R1-forward primer (bases 275 to 294), and (2) a composite 37-mer reverse primer made up of the 19-mer CRH R1-reverse primer described above (bases 866 to 848) with an additional 18 bases (773 to 756 of CRH R1) at its 3' end. A schematic representation is shown in Fig. 1B.

When using competitive PCR it is important also to measure a housekeeping gene as a control for variation in the quality and quantity of RNA. The constitutive GCB gene (Sorge *et al.* 1985) has been used for this purpose (Chambers & Harris 1993). A 509 bp internal cDNA standard for GCB was made from cDNA by PCR using the following primers: (1) the 20-mer GCB-forward primer (bases 1182 to 1201), and (2) a composite 40-mer reverse primer made up of the 20-mer GCB-reverse primer described above (bases 1753 to 1734) with an additional 20 bases (1670 to 1651 of GCB) at its 3' end.

The cDNA standards were purified with the QIAquick PCR purification kit (Qiagen Ltd, Crawley, W. Sussex, UK), quantified by spectrophotometry, and diluted to 100 attomoles/ μ l in 50 μ g glycogen/ml. Initially, target cDNA was titrated in the first series of competitive PCR assays: a constant amount of RT product (0.1 μ g total RNA/ μ l) was mixed with consecutive 1:10 dilutions of the cDNA standard down to 0.0001 attomoles/ μ l. A second series of competitive PCR assays was carried out with consecutive 1:2 dilutions of the cDNA standard mixed with the constant amount of target cDNA described above. The result of one

FIGURE 2. Schematic representation of a QC-PCR experiment for the CRH R1. (A) Myometrial mRNA from two non-pregnant patients was reverse transcribed and the resulting cDNA was used as a template for PCR amplification in the presence of 2-fold serial dilutions of the homologous cDNA competitor fragment (518 bp). The CRH R1 fragment is 592 bp in size. The samples were run on a 1.5% agarose gel and stained with ethidium bromide. (B) The relative intensities of the bands corresponding to the target and the competitor were quantified by computer imaging of the Polaroid photo of the gel (from panel A) and the logarithm of the ratio of amplified target to cDNA standard is plotted as a function of the logarithm of the known amount of competitor added to the PCR reaction. Linear regression analysis of the data points shows the amount of competitor needed to produce an equimolar amount of target.

experiment using 1:2 dilutions of the cDNA standard for the CRH R1 receptor on non-pregnant myometrium from two different patients is shown in Fig. 2A. The PCR reactions were carried out under the same conditions as described in the 'RNA isolation and RT-PCR amplification' section above. Transcribed RNA from each patient was assayed at least three times. Samples were loaded on 1.5% agarose gels containing 1 μ g ethidium bromide per ml, and photographed under UV illumination. The

TABLE 1. Patient details giving the age of each patient and the duration of their labour in hours. Each patient is designated by an arbitrary number code. The reason for the caesarean section performed in labouring patients is also indicated. FD, fetal distress; FP, failure to progress

Non-pregnant		Not in labour		Labour				
Code	Age (years)	Code	Age (years)	Code	Age (years)	Duration (h)	Indication	Cervical dilatation (cm)
#501	42	#527	23	#524	31	4	FD	2
#502	36	#535	28	#560	29	2	FD	8
#503	43	#537	24	#572	34	13	FP	4
#504	40	#538	30	#577	29	9	FP	4
#505	34	#542	26	#583	30	1·5	FD	4
		#544	23	#590	27	11	FD	4
		#545	38	#607	36	13	FP	5
				#621	32	4	FD	2

bands were quantified by scanning densitometry and the use of Intelligent Quantifier software (BioImage, Ann Arbor, MI, USA). The logarithm of the ratio of amplified target to cDNA standard was plotted as a function of the logarithm of the known amount of cDNA standard present in the PCR reaction. The amount of cDNA standard necessary to produce an equimolar amount of target was calculated through linear regression analysis of the data points and expressed as attomoles/ μ g total RNA. Using this method and using Fig. 2 as an example, we have calculated that patient #501 had 0·015 attomoles CRH R1 mRNA per μ g total RNA while patient #502, represented in Fig. 2B, had 0·035 attomoles per μ g total RNA. After correction for the size difference between the cDNA standard and the target PCR products, levels of CRH R1 mRNA were normalised with the expression of the housekeeping gene GCB and expressed as a ratio of CRH R1:GCB. Statistical analysis was performed using the non-parametric one-way ANOVA (Kruskal–Wallis) for comparison between the different groups.

RESULTS

Competitive PCR analysis

Initially, we investigated the validity of the competitive PCR analysis by using a known quantity of cDNA. A known amount of the CRH R1 592 bp fragment synthesised by a previous PCR (0·01 attomoles) was added to a PCR reaction, together with different concentrations of the cDNA standard for the CRH R1 receptor (0·16–0·00125 attomoles). The equivalence point was found to be at 0·007 $\pm$

0·001 attomoles, in close agreement with the amount of cDNA added to the reaction.

CRH R1 expression in pregnancy at labour

Using competitive PCR, the level of CRH R1 mRNA in the AtT20/D16-16 cell line included as a positive control (Chen *et al.* 1993, Vita *et al.* 1993) was measured as 1·75 attomoles/ μ g total RNA.

We used this technique to measure the levels of CRH R1 gene expression in human myometrium from three groups of women: non-pregnant ($n=5$), pregnant at term but not in labour ($n=7$), and pregnant at term in spontaneous labour ($n=8$). Details for each individual are shown in Table 1. Figure 3A shows the levels of CRH R1 mRNA expressed as attomoles/ μ g total RNA. Statistical analysis of these data showed no significant differences between the three groups ($P>0\cdot05$). For comparative purposes, levels of oxytocin receptor (OTR) mRNA in 13 pregnant women at term within 12 h of the onset of spontaneous labour were also measured (Phaneuf *et al.* 1998). The OTR mRNA levels were 69 ± 22 attomoles/ μ g total RNA, whereas those of CRH R1 were significantly lower ($0\cdot06 \pm 0\cdot01$ attomoles/ μ g total RNA).

The level of expression of the housekeeping gene GCB was measured as $0\cdot70 \pm 0\cdot18$, $2\cdot72 \pm 0\cdot67$ and $4\cdot72 \pm 0\cdot69$ attomoles/ μ g total RNA in the same samples of the non-pregnant, not in labour and spontaneous labour groups respectively. Although GCB mRNA demonstrated a tendency to increase in these successive groups, there were no statistical differences between the non-pregnant and not in labour groups and, most interestingly, between the non-labour and spontaneous labour groups ($P>0\cdot05$).

FIGURE 3. (A) CRH R1 mRNA levels in the myometrium from the three groups of women (NP, non-pregnant, NIL, not in labour, SL, spontaneous labour). Myometrial mRNA was reverse transcribed and the resulting cDNA was used as a template for PCR amplification in the presence of decreasing amounts of the homologous cDNA competitor fragment. After imaging analysis of the bands and linear regression analysis, data were expressed as attomoles/μg total RNA. (B) Ratio of CRH R1:GCB mRNA levels in the myometrium from the three groups of women. CRH R1 mRNA levels (attomoles/μg total RNA) for each patient were normalised with the expression of the housekeeping gene GCB in attomoles/μg total RNA. (C) Levels of CRH R1 during the progress of labour. The ratio of CRH R1:GCB mRNA levels was measured in the myometrium from not in labour women and women in spontaneous labour (FD, fetal distress, FP, failure to progress). The spontaneous labour samples are plotted against the time of sampling in labour and analysed by linear regression, $r^2=0.065$.

Figure 3B shows that, when the data were expressed as a ratio of CRH R1:GCB mRNA, myometrium from pregnant patients at term but not in labour contains lower levels of CRH R1 than myometrium from non-pregnant women (0.012 ± 0.005 compared with 0.093 ± 0.011 , $P < 0.01$). This figure also shows that there is no further change in this ratio in women in spontaneous labour (0.013 ± 0.004 , $P > 0.05$).

As shown in Fig. 3C, there is no correlation between CRH R1 gene expression and the duration of labour when measured as the ratio of CRH R1:GCB mRNA. Furthermore, there were no statistical differences between samples obtained from patients who had a caesarean section for 'fetal distress' and those obtained for 'failure to progress' and the duration of labour.

DISCUSSION

In the present study using competitive PCR, we have measured the expression of CRH R1

mRNA in myometrium from non-pregnant and pregnant women before and after the onset of labour. Once normalised, the data show that there is an 8-fold decrease in the level of the CRH R1 in pregnancy and that myometrial CRH R1 levels remain low during parturition. There is a tendency (although not significant) for the GCB housekeeping gene mRNA to increase in pregnancy and labour, probably due to the extreme sensitivity of competitive PCR and to the structural changes in stretched myocytes in late pregnancy. Other traditionally used housekeeping genes such as actin were considered unsuitable for the study carried out here, since it has been shown that actin expression is modified under the challenge of different mitogenic agents (Savonet *et al.* 1997). Another popular housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was also rejected, as GAPDH expression is known to increase in uterine artery endothelial cells during pregnancy (Cale *et al.* 1997). Moreover, a recent study shows that β -actin and GAPDH are

not constitutively expressed in human gestational tissues (Freed *et al.* 1997).

From our findings it seems unlikely that CRH acting on the R1 receptor participates directly in uterine contractility at term. The low levels of CRH R1 at term and during labour contrast dramatically with that of OTR, which is known to have a direct role in the stimulation of uterine contractility in labour. Indeed, as shown in the present study there is a 1000-fold difference between the mRNA levels of OTR and CRH R1 in myometrium from patients in spontaneous labour. Moreover, myometrial OTR and its mRNA increase during pregnancy to reach levels at term considerably higher than those found in non-pregnant tissue (Fuchs *et al.* 1984, Kimura *et al.* 1996). Our results agree with the absence of an effect of CRH on uterotonic-induced contractions *in vitro* (Simpkin *et al.* 1997). Moreover, our data showing the reduced expression of CRH R1 in pregnant tissue at term agree well with the decrease in CRH-induced cAMP production at the end of pregnancy reported by Grammatopoulos *et al.* (1996). The results from both studies might be explained in terms of a marked reduction of receptor gene expression in term human myometrium, as CRH down-regulates its own receptors in rat anterior pituitary cell cultures by decreasing mRNA levels (Pozzoli *et al.* 1996). It is interesting to note that at term, maternal plasma levels of free circulating CRH are at their highest; the decrease in CRH R1 levels at term compared with non-pregnant myometrium observed in this study could result from the down-regulatory effect of increasing plasma levels of CRH on the CRH R1 receptor. Further work with pure cultures of uterine myocytes is required to test this hypothesis.

Our data were obtained from pregnant myometrial samples taken from the lower segment, which may be less contractile than the fundus. Although there is no direct evidence for regional variation in contraction in the human pregnant uterus, there is evidence that some receptors are differentially distributed. It has been shown, for example, that OTR density follows a gradient from high levels in the fundus to lower levels near the cervix (Fuchs *et al.* 1984, López Bernal *et al.* 1995). For ethical reasons we do not have routine access to samples from the corpus and fundus, and at present there are no data available on the regional distribution of CRH receptors in the uterus.

Taken together, our results and the available evidence favour the suggestion that CRH participates in myometrial quiescence during pregnancy. To investigate this hypothesis, measurement of

CRH R1 mRNA and protein levels in samples of tissue obtained in early pregnancy would be of interest, although the availability of such tissues is severely limited. Our data do not exclude an indirect participation of CRH in PG-induced myometrial contractility. It has been shown that CRH stimulates PGE₂ release from cultured human decidual cells (Petraglia *et al.* 1995) and induces PGE₂ and PGF_{2α} synthesis in human fetal membranes and placenta (Jones & Challis 1989). In contrast with the present findings, recent preliminary work (Stevens *et al.* 1997) suggests that there is an up-regulation of the CRH R1 in labouring myometrium. The discrepancy with our results could be due to differences in technical approach. Our more extensive investigation also indicates that there is no correlation between the duration of labour and the level of expression of the CRH R1.

The highest levels of gene expression of the CRH R1 measured in non-pregnant myometrium suggest that CRH might play a role in this tissue. There is increasing evidence for an autocrine/paracrine role of CRH in the inflammatory processes associated with the menstrual cycle in the uterus: this hormone is present in non-pregnant myometrium (Clifton *et al.* 1996), CRH and the gene for the CRH R1 are present in endometrial stromal cells (Mastorakos *et al.* 1996, Di Blasio *et al.* 1997), and CRH has been shown to induce the decidualisation of endometrial stromal cells *in vitro* (Ferrari *et al.* 1995).

In summary, we have found that there is a down-regulation in the levels of myometrial CRH R1 with pregnancy measured as the ratio of CRH R1:GCB mRNA. The lack of an up-regulation of the receptor at term and during labour, together with the lack of a functional effect of CRH on myometrium found by others, does not support a direct role for the CRH R1 in myometrial contractility. Our results do not rule out the involvement of other subtypes of CRH receptors not yet characterised. The involvement of CRH receptors in myometrial stimulation remains controversial and requires further investigation.

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