Role of stress peptides during human pregnancy and labour

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Premature birth is the major source of perinatal death and disability. Furthermore, the intrauterine health of the baby is important for preventing certain adult diseases. However, the molecular mechanisms driving the onset of human labour remain uncertain, although several key players have been identified. It is becoming clear that there are many pathways to parturition in humans. Stress peptides, in particular placental corticotrophin releasing hormone (CRH) and possibly the related peptide urocortin, appear to play important roles throughout pregnancy. Plasma CRH is a predictor of the duration of human gestation. During most of pregnancy, CRH, acting via specific CRH receptor subtypes, plays a 'protective' role by promoting myometrial quiescence via the generation of cAMP and cGMP, and upregulation of nitric oxide synthase expression. At term, myometrial contractility is enhanced by a complex series of molecular switches, involving the upregulation of oxytocin receptor expression and crosstalk between the oxytocin and CRH receptors. This results in protein kinase C-induced phosphorylation of specific CRH receptor subtypes, with subsequent desensitization and a shift in the intracellular microenvironment to enhance contractility. CRH/urocortin, via specific receptor isoforms, is now able to activate Gq and potentially enhance the oxytocin-driven generation of inositol triphosphate. In addition, CRH/urocortin, via specific CRH receptor subtypes, may generate prostaglandins from the fetal membranes and decidua, play a role in placental vasodilatation and participate in fetal adrenal function and organ maturation. These peptides and receptors are phylogenetically ancient and well preserved across species. They may have evolved as a mechanism to protect against the 'stress' of premature birth.

During gestation, the uterus remains in a state of quiescence to ensure successful maturation of the fetus. At term, the cervix dilates and the uterus switches from quiescence to a state of co-ordinated contractility. The molecular mechanisms underlying these complex physiological events remain elusive. In up to 10% of human pregnancies, the mechanisms controlling these processes are altered in such a way that pre-term birth takes place. Although parturition in humans appears to be unique, there are features that are preserved across species. One such feature is the activation of the maternal and fetal hypothalamo–pituitary–adrenal axes, the primary function of which is to control the response of the body to stress (Liggins, 1976; Thorburn and Challis, 1979; Challis *et al.*, 2000). A key component of this axis is corticotrophin releasing hormone (CRH), a 41 amino acid hypothalamic peptide that helps to co-ordinate the endocrine, autonomic, behavioural, cardiovascular and immune responses to stress. Recently, it has become clear that CRH is part of a larger family of stress-related peptides

that includes the urocortins (Vaughan *et al.*, 1995; Donaldson *et al.*, 1996; Lewis *et al.*, 2001; Reyes *et al.*, 2001). These related peptides mediate their effects on the target cells via seven helical transmembrane domain receptors that transduce their signal through stimulation of various G proteins.

Plasma CRH and urocortin

CRH is synthesized by fetomaternal tissues (Petraglia *et al.*, 1987; Riley *et al.*, 1991; Warren and Silverman, 1995; Clifton *et al.*, 1998; Gravanis *et al.*, 2001) and is secreted into the maternal circulation during gestation, where concentrations increase exponentially as pregnancy progresses (Goland *et al.*, 1986; Campbell *et al.*, 1987). It has been suggested that there is a 'CRH placental clock' which is active from the early stages of human pregnancy and determines the duration of gestation and the timing of parturition and delivery (McLean *et al.*, 1995). Interestingly, patients presenting in pre-term labour have high concentrations of circulating CRH (Campbell *et al.*, 1987; Sasaki *et al.*, 1987; Warren *et al.*, 1992). The utility of plasma CRH measurement in the prediction of pre-term labour has not

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yet been fully elucidated. However, it can be used to identify patients presenting in pre-term labour who will deliver within the next 24–48 h (Korebrits *et al.*, 1998). In the maternal circulation, CRH attaches to a binding protein (Behan *et al.*, 1989; Potter *et al.*, 1991) and circulates as a dimerized binding protein–ligand complex. Patients at risk of undergoing pre-term labour have significantly increased maternal concentrations of CRH, lower concentrations of CRH binding protein and a reduction in the CRH binding protein–CRH dimer complex (Hobel *et al.*, 1999). Thus, free concentrations of CRH are increased in the maternal circulation and may play a role in the pathophysiological events leading to pre-term labour. Urocortin is also expressed in human placenta, fetal membranes and uterine tissues (Petraglia *et al.*, 1996; Clifton *et al.*, 2000). Urocortin is detectable at low concentrations in maternal plasma from several weeks of gestation but the concentrations do not change during gestation (Clifton *et al.*, 2000).

The placental expression of the CRH gene and secretion of biologically active CRH is under the control of a number of factors including nitric oxide, progesterone, catecholamines, oxytocin, cytokines and glucocorticoids (Challis *et al.*, 2000). The key interaction appears to be that between cortisol and progesterone, as the latter inhibits CRH gene expression, an effect that can be reversed by cortisol (Karalis *et al.*, 1996).

Possible roles of CRH and related peptides during pregnancy

The precise biological functions of CRH and related peptides during pregnancy are unknown. However, it is known that a series of molecular events mediated by autocrine, endocrine and paracrine actions of CRH, and possibly urocortin, is activated during pregnancy and at term to prepare the fetus and uterus for parturition. A number of hypotheses, which are not mutually exclusive, have emerged: (i) CRH and urocortin may regulate myometrial contractility/quiescence via a direct action on myometrial cells and, thus, play a role in the prevention of premature labour and in the onset of term labour (Grammatopoulos and Hillhouse, 1999); (ii) CRH and urocortin may influence myometrial contractility indirectly; for example, CRH stimulates prostaglandin production by the fetal membranes and placenta (Jones and Challis, 1989); (iii) CRH or urocortin or both may play a role in regulation of placental blood flow (Clifton *et al.*, 1995; Leitch *et al.*, 1998). The vasodilatory effects of both peptides appear to be mediated by a nitric oxide/cGMP-dependent pathway with urocortin being the most potent vasodilator; and (iv) CRH may play an important role in the regulation of fetal adrenal function and, hence, maturation of various organ systems (Randeva *et al.*, 2001). These mechanisms may have evolved to protect the fetus against environmental stress. However, under certain circumstances, it is possible that fetal or maternal stress could play an important role in

initiating a cascade of intracellular signals leading to pre-term labour.

The CRH receptor family

The CRH-related family of peptides exert physiological responses that are co-ordinated by the expression of two receptors, CRHR1 (Chen *et al.*, 1993) and CRHR2 (Lovenberg *et al.*, 1995), encoded by separate genes mapped to human chromosomes 17 (Polymeropoulos *et al.*, 1995) and 7 (Meyer *et al.*, 1997), respectively. These receptors belong to the group II subfamily of seven helical transmembrane spanning domain G protein-coupled receptors, which includes receptors for calcitonin, vasoactive intestinal peptide and parathyroid hormone. The CRHR1 binds CRH and related peptides (urocortin, urotensin and sauvagine) with high affinity. CRHR2 binds urocortin with higher affinity than the other CRH-like peptides and also binds the newly identified urocortin 2 and urocortin 3 peptides, indicating that these may be the natural or preferred ligands (Vaughan *et al.*, 1995; Lewis *et al.*, 2001; Reyes *et al.*, 2001). CRHR1 shares 70% similarity with CRHR2 and both receptors are present in structurally distinct isoforms. The CRHR1 gene expresses four known subtypes, 1 α , 1 β , 1c and 1d (for references, see Grammatopoulos and Hillhouse, 1999; Grammatopoulos *et al.*, 1999), produced by differential splicing of exons 3, 6 and 13 (Fig. 1). The CRHR2 gene expresses three known subtypes, 2 β , 2 γ and 2 α (for references, see Hillhouse and Grammatopoulos, 2001), that are produced by the use of alternate 5' exons (Fig. 2) and, hence, differ only at the N terminus that forms part of the first extracellular domain. The expression of type I CRH receptors is controlled by a single promoter and use of differential splicing, whereas the type 2 receptor uses multiple promoters in addition to alternative splicing (R. Catalano, D. K. Grammatopoulos and E. W. Hillhouse, unpublished).

Expression of CRH receptors in fetomaternal tissues

Non-pregnant human myometrium expresses three CRH receptor subtypes, namely 1 α , 1 β and 2 β . As pregnancy progresses, the myometrium starts to express the 2 α receptor. In addition, at term the myometrium expresses the 1c and 1d receptor subtypes (Grammatopoulos *et al.*, 1998, 2000), indicating a possible functional role for these receptor subtypes at the end of pregnancy. The molecular mechanisms regulating the expression of the receptor subtypes remain unknown but presumably involve fine interplay between the gene promoters and the mRNA splicing enzymes. Furthermore, these observations imply that the biological actions of CRH and urocortin in myometrium during the different stages of pregnancy are mediated via different CRH receptor subtypes. The syncytiotrophoblasts of the placenta and the fetal membranes express the 1 α , 1c, 1d and 2 β subtypes (Karteris *et al.*, 1998; Florio *et al.*, 2000), and the fetal adrenal glands express both the 1 α and 2 α subtypes (Smith *et al.*, 1998; Karteris *et al.*, 2001).

Fig. 1. Schematic representation of the mRNA encoding corticotrophin releasing hormone receptor 1 α (CRHR1 α). Different receptor isoforms are generated by alternative exon splicing. SP: signal peptide; TM: transmembrane domain; EC: extracellular domain; il: intracellular loop; el: extracellular loop; IC-tail: intracellular tail; aa: amino acids.

Fig. 2. (a) Schematic representation of the human corticotrophin releasing hormone receptor 2 (CRHR2) gene. Exons are indicated by purple boxes and 5'-3' untranslated regions are indicated by yellow boxes. The CRHR2 gene spans 50 kb and consists of 15 exons. The distances between the exons are drawn to scale but not in relation to exon size. The β , γ and α receptors. (b) Confirmed non-functional transcripts arising from aberrant splicing.

Roles of CRH and urocortin in the control of myometrial contractility during pregnancy

In human myometrium, CRH activates multiple classes of G protein, namely G α_s , G α_i , G α_o , G α_q and G α_z , indicating

that it may signal via multiple signalling pathways. To date, our work has focused on the G α_s and G α_q systems. In the non-pregnant state, the CRH receptors do not couple to the adenylate cyclase system (Grammatopoulos *et al.*, 1994). However, during pregnancy, there is coupling

to $G_{s\alpha}$ and the adenylate cyclase system leading to production of cAMP that shifts the balance of the intracellular microenvironment towards myometrial quiescence. This effect of CRH is reinforced by stimulation of other intracellular signalling pathways that are also involved in the relaxation of myometrium during pregnancy. For example, CRH activates membrane-bound guanyl cyclase via a protein kinase A (PKA)-dependent mechanism leading to production of cGMP and activation of the nitric oxide system. In addition, CRH stimulates activity of the soluble form of guanyl cyclase via a PKA-independent mechanism that involves upregulation of the constitutive form of nitric oxide synthase (Aggelidou *et al.*, 2002). Furthermore, CRH can block the cytokine-induced activation of myometrial prostaglandin production (Grammatopoulos and Hillhouse, 1999) that has been proposed as a mechanism for infection-induced pre-term labour (Pollard and Mitchell, 1996).

In pre-term myometrium, CRH activates both the short (45 kDa) and the long (52 kDa) form of the $G_{s\alpha}$ protein, whereas it has no effect on $G_{q\alpha}$ protein activation. This pattern of G protein activation favours generation of intracellular cAMP and promotion of myometrial quiescence. However, at term a significant change takes place; CRH now activates only the short form of $G_{s\alpha}$ protein but, more importantly, also activates the $G_{q\alpha}$ protein (H. Randeva, D. K. Grammatopoulos and E. W. Hillhouse, unpublished). The precise function of the different isoforms of the $G_{s\alpha}$ proteins remains poorly characterized and, therefore, the exact physiological significance of these events remains to be determined. However, it would seem likely that before term, CRH plays a 'protective' role for the myometrium by preventing uterine contractions. If this is true, then the increased concentrations of maternal circulating CRH in abnormal pregnancy states (Goland *et al.*, 1995) might be a response to an abnormal or premature labouring process to prevent the development of premature contractions and protect the fetus from expulsion. Activation of the $G_{q\alpha}$ protein with subsequent stimulation of the phospholipase C/inositol triphosphate pathway supports the contention that there is a shift in the role of CRH towards term to enhance myometrial contractility and labour. However, it is important to realise that these data have been obtained using biopsies from the lower segment of the uterus and that there may be region-specific changes in CRH receptor subtypes in the uterus during pregnancy and labour. This may be particularly important in the fundus, which plays an essential role in uterine activity and contractions.

Myometrial intracellular crosstalk between oxytocin and CRH receptors

Oxytocin is a powerful uterotonic and is thought to play a role in the mechanisms leading to parturition. However, this view has been questioned by observations of mice in which the oxytocin gene was inactivated by homologous recombination. These studies showed that oxytocin is

essential for lactation but not for normal parturition in mice (Nishimori *et al.*, 1996; Young *et al.*, 1996). However, in humans the oxytocin system appears to participate in the mechanisms driving normal and abnormal labour, as the oxytocin receptor antagonist Atosiban induces uterine quiescence in both normal and pre-term labour (Romero *et al.*, 2000; Valenzuela *et al.*, 2000). Further support for this view is provided by the observations of a marked up-regulation of uterine oxytocin receptors before term (Fuchs *et al.*, 1982). The oxytocin receptor gene may form part of a gene 'cassette' (Lefebvre *et al.*, 1995), induction of which is essential for the successful initiation of labour. An important advance in our understanding of the role of oxytocin in parturition was the demonstration that the decidua is a major site of oxytocin gene expression (Lefebvre *et al.*, 1992; Chibbar *et al.*, 1993), raising the possibility of paracrine effects of oxytocin. However, in humans, only the mRNA has been identified and so paracrine effects remain speculative.

The oxytocin receptor couples to $G_{q\alpha}$ proteins and the inositol triphosphate pathway, leading to increases in intracellular calcium and phosphorylation of the myosin light chains. In addition, the oxytocin receptor can activate MAPK (Ohmichi *et al.*, 1995; Molnar *et al.*, 1999). Thus, activation of the oxytocin receptor leads to a shift in the intracellular microenvironment from quiescence to contractility. This effect is amplified by important intracellular crosstalk between the oxytocin and CRH receptors. At term, but not pre-term, oxytocin activates protein kinase C (PKC), which phosphorylates the CRH receptor at one or more specific Ser or Thr residues, resulting in receptor desensitization and reduction in adenylate cyclase activation (Grammatopoulos *et al.*, 1996; Grammatopoulos and Hillhouse, 1999). In addition, activation of PKC results in a reduction in membrane-bound guanyl cyclase activity and cGMP production, thereby neutralizing relaxation pathways at multiple levels. This activation of PKC occurs only in term myometrium, possibly due to the increased number of oxytocin receptors. Recently, we have been able to show that this PKC-induced desensitization is mediated predominantly via the CRH-1 β receptor subtypes (D. K. Grammatopoulos, M. Levine and E. W. Hillhouse, unpublished).

At term, CRH or urocortin or both may enhance myometrial contractility, as both augment the myometrial contractile response to $PGF_{2\alpha}$ and oxytocin (Benedetto *et al.*, 1994; Petraglia *et al.*, 1999). Furthermore, urocortin promotes myometrial contractility via activation of the MAPK signalling pathways, an effect mediated via activation of the 1 α and 2 β receptor subtypes (Grammatopoulos *et al.*, 2000). These observations are indicative of a dual role for CRH or urocortin or both. Such a mechanism would enable these peptides to promote myometrial relaxation during most of gestation but to enhance the contractile response of the myometrium at term. This model places CRH in a central role in co-ordinating the smooth transition from a state of quiescence to one of contractility.

Fig. 3. Proposed signalling cascades activated by corticotrophin releasing hormone (CRH) ligands in fetomaternal tissues during human pregnancy. CRHR: CRH receptor; NOS: nitric oxide synthase; PG: prostaglandin; MAPK: MAP kinase; ACTH: adrenocorticotrophic hormone.

Role of CRH and related peptides in placenta, fetal membranes and adrenal glands

In the placenta and fetal membranes, CRH does not signal via adenylate cyclase; however, it can activate G α and G q in both tissues, and also G α in the placenta (Karteris *et al.*, 2000). In addition, CRH stimulates the production of inositol triphosphates (Karteris *et al.*, 2000), which may act as a signal to prostaglandin biosynthesis secondary to induction of type 2 cyclo-oxygenase (Jones and Challis, 1989). This is significant as β agonists that activate adenyl cyclase and generate cAMP in these tissues also upregulate COX 2 expression (Warrick *et al.*, 1985). This dichotomy may prove to have an important functional consequence but at the moment this remains elusive. In the placenta, CRH also stimulates the production of adrenocorticotrophic hormone (ACTH) in an analogous manner to that in the pituitary gland, but not via activation of cAMP (Petraglia *et al.*, 1987).

CRH and urocortin also play an important role in regulating blood flow in the placenta via activation of the nitric oxide-cGMP system (Clifton *et al.*, 1995; Leitch *et al.*,

1998). This effect may be mediated via activation of type 2 receptors, as urocortin is more potent than CRH in inducing placental vasodilatation. This is in contrast to the myometrium in which CRH also activates the nitric oxide-cGMP system via type 1 receptors and promotes myometrial relaxation (Aggelidou *et al.*, 2002).

In the fetal adrenal gland, CRH stimulates P450c17 activity via generation of inositol triphosphates leading to dihydroepiandrosterone production (Smith *et al.*, 1998; Chakravorty *et al.*, 1999). The latter is the principal substrate for placental oestrogen synthesis, which has a profound influence on CRH receptor subtype expression (E. W. Hillhouse and D. K. Grammatopoulos, unpublished). Thus it is possible that CRH itself may indirectly influence tissue expression of CRH receptors and, hence, its own biological actions during gestation. Whether the effects of oestrogen are due to actions on promoter activity, splicing enzymes or a combination of the two is currently unknown. Oestrogen also has profound effects on myometrial G protein expression and generation of intracellular second messengers (Zervou *et al.*, 2002).

Conclusion

During pregnancy and labour there is a progressive increase in maternal plasma concentrations of CRH, which can be used as a marker of pre-term delivery. This could be interpreted as CRH driving mechanisms leading to premature birth; however, the available evidence suggests the opposite. It appears likely that CRH, acting via specific CRH receptor subtypes, dampens myometrial contractility during most of gestation (Fig. 3). Thus, CRH acts as a 'stress peptide' that is synthesized in increased amounts in 'at risk' pregnancies to try to prevent premature myometrial contractions. However, at term, these 'protective mechanisms' are disabled under the influence of the oxytocin receptor, thereby allowing CRH, via alternate specific receptor subtypes, to participate in the mechanisms leading to expulsion of the fetus.

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