Corticotrophin-releasing hormone inhibits insulin-like growth factor-I release from primary cultures of rat granulosa cells

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Abstract

Corticotrophin-releasing hormone (CRH), a neuropeptide which modulates gonadal function during stress, is expressed by several cell types of the rat ovary and is able to suppress oestrogen release from rat granulosa cells. The mechanism of this effect is, however, not known. Since insulin-like growth factor (IGF)-I is produced by rat granulosa cells and exerts a synergistic role with FSH on granulosa cell steroidogenesis, we hypothesised that CRH may suppress oestrogen release from granulosa cells by inhibiting IGF-I release and/or stimulating the release of its binding protein (IGFBP-3). To test this hypothesis, granulosa cells were obtained from immature female Sprague-Dawley rats primed with diethylstilboestrol, and hormone concentrations were measured in the conditioned medium by radioimmunoassay. CRH suppressed oestrogen and IGF-I release stimulated by FSH used at a

concentration of 1 IU/l, whereas it did not have any statistically significant effect on oestrogen and IGF-I release in basal conditions or in response to 5 IU/l FSH. The suppressive effects of CRH on oestrogen and IGF-I release were antagonised by a selective CRH receptor antagonist. CRH had no effects on IGFBP-3 release. CRH did not have any effect on oestrogen release stimulated by increasing concentrations of IGF-I and its suppressive effect on FSH-stimulated oestrogen release was overcome by the addition of low doses of exogenous IGF-I. In conclusion, CRH suppressed the release of oestrogen and IGF-I, but not of IGFBP-3. Thus, the inhibitory effects of CRH on oestrogen release could be mediated, partly, by a suppression of the autocrine/paracrine action of IGF-I.

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Introduction

Corticotrophin-releasing hormone (CRH), a 41-amino acid peptide secreted by the paraventricular nucleus of the hypothalamus, is the main activator of the hypothalamic–pituitary–adrenal axis and it exerts a major role in coordinating the endocrine, autonomic and behavioural responses to stress (Chrousos & Gold 1992). This neuropeptide also plays an important role in the suppression of the hypothalamic–pituitary–gonadal (HPG) axis during stressful events (see Rivier & Rivest 1991 for review). Indeed, CRH has been shown to be capable of inhibiting the release of gonadotrophin-releasing hormone at the hypothalamic level (see Calogero *et al.* 1998 for review). This is not, however, the only mechanism through which this neuropeptide inhibits the function of the HPG axis. Experimental evidence suggests that CRH may also act as a local anti-reproductive factor in the male rat (Yoon *et al.* 1988, Ulisse *et al.* 1989). More recent findings have shown that CRH is also produced by the ovary where it is

localised in theca cells surrounding follicles, stroma cells, mature oocytes within antral follicles and ovarian resident macrophages. In the developing corpora lutea, it is detectable in the cytoplasm of both small theca-derived and large granulosa-derived luteinized cells (Mastorakos *et al.* 1993, Muglia *et al.* 1994, Asakura *et al.* 1997). It has been shown to restrain the production of oestrogen by rat and human granulosa cells (Calogero *et al.* 1996, Ghizzoni *et al.* 1997). The mechanism(s) of this inhibition is, however, not well known.

In recent years, a large body of literature has highlighted the importance of insulin-like growth factors (IGFs) in regulating the development and the function of ovarian follicles under the modulatory effects of IGF binding proteins (IGFBPs). It has been shown that all components of the system are synthesised in ovarian follicles, thus revealing that IGFs and IGFBPs can act through an autocrine/paracrine mechanism in addition to the classical endocrine mechanism (see Giudice 1992 for review). IGF-I, a basic member of this family, is produced by the

granulosa cells of the developing follicle in the rat (Oliver *et al.* 1989). In murine ovaries, besides a potent stimulatory effect on granulosa cell replication (Zhou *et al.* 1991), IGF-I also exerts a stimulatory effect on the synthesis of proteoglycans (Adashi *et al.* 1986) and on aromatase activity (Adashi *et al.* 1985a). Moreover, it has a synergistic role with follicle-stimulating hormone (FSH) on steroidogenesis and luteinizing hormone receptor induction (Adashi *et al.* 1985b,c), probably by stimulating FSH receptor gene expression in granulosa cells (Zhou *et al.* 1997). The fact that, using an *in situ* hybridisation technique, the mRNAs for IGFBP-2, -3 and -4 have been localised in the interstitial cells, corpora lutea, and atretic granulosa cells respectively, hints at a different regulation for each of these IGFBPs, which may have a role as autocrine/paracrine factors in modulating the local actions of the IGFs in the rat ovary (Nakatani *et al.* 1991). The effect of FSH on the production of IGFBPs by granulosa cells from immature oestrogen-primed rats has been shown to be dose- and time-dependent (Adashi *et al.* 1991). Indeed, high levels of FSH inhibit the local synthesis/release of the IGFBPs which, in turn, have an inhibitory effect on IGF biological activity (Adashi *et al.* 1990, Barreca *et al.* 1996, Mason *et al.* 1998). The consequence is a great potentiation of the biological effect of FSH on follicular maturation.

The antithetical effects of CRH and IGF-I (Adashi *et al.* 1985a, Calogero *et al.* 1996) at the ovarian level led us to hypothesise that the suppressive effects of CRH on oestrogen release from granulosa cells may be due to an inhibition of IGF-I production and/or a stimulation of IGFBP-3 production. To test this hypothesis, we studied the effects of CRH on IGF-I and IGFBP-3 release into the conditioned medium of primary cultures of rat granulosa cells. We also evaluated the effect of increasing concentrations of CRH on IGF-I-stimulated oestrogen release.

Materials and Methods

Rat granulosa cell culture

Rat granulosa cells were cultured as previously reported (Calogero *et al.* 1996) with slight modifications. Briefly, intact female immature (25 days old) Sprague-Dawley rats (Charles River, Calco, CO, Italy) were implanted subcutaneously with 10-mm silastic capsules filled with diethylstilboestrol (DES) (Sigma Chemical Co., St Louis, MO, USA). Four days after the implantation, rats were killed by cervical dislocation and ovaries were removed for granulosa cell collection. Ovaries were decapsulated, follicles were punctured with 27-gauge hypodermic needles, and granulosa cells were expressed into McCoy's 5a medium (Life Technologies, Paisley, Strathclyde, UK) to make a single cell suspension which was used for control and experimental cultures. An aliquot was diluted with

trypan blue stain and viable cells were counted with a haemocytometer. Cells were incubated in McCoy's 5a medium supplemented with 2 mmol/l L-glutamine, 200 U/ml penicillin, 200 µg/ml streptomycin sulphate, 0.5 µg/ml fungizone (Life Technologies), 10^{-6} mol/l androstenedione, 10^{-7} mol/l DES, 35 µg/ml methylisobutylxanthine (Sigma), and 37.5 ng/ml human chorionic gonadotrophin (hCG) (CR-127, NIDDK, Bethesda, MD, USA). Cells were cultured for 6 days in a humidified 95% air-5% CO₂ incubator at 37 °C with the following treatments: medium alone (control), medium containing human FSH (NIDDK, hFSH B-1, AFP-8792B), rat/human CRH (Peninsula Laboratories Europe Ltd, St Helens, Merseyside, UK), α -helical CRH₉₋₄₁ (Peninsula Laboratories), and/or human recombinant IGF-I (Amersham International plc, Amersham Bucks, UK). To evaluate the effects of CRH on IGF-I and IGFBP-3 release, granulosa cells were plated onto 25-cm² flasks (Corning, Milan, Italy), at a density of 10^6 cells/ml (10 ml/flask). CRH was used at a concentration of 10^{-7} mol/l, a concentration known to be capable of suppressing FSH (1 IU/l)-stimulated oestrogen release by about 30% (Calogero *et al.* 1996). Each experiment was performed in at least six different cultures. To evaluate the effects of IGF-I, FSH and/or CRH on oestrogen release, granulosa cells were plated onto 16-mm, 24-multiwell plates (Corning), at a density of 50 000 cells/well/0.5 ml. Each experiment was performed in four different cultures. At the end of the culture period, the incubation medium was collected and centrifuged. The supernatant was stored at -20 °C until assayed for IGF-I, IGFBP-3 and oestrogen concentration.

Oestrogen measurement

The concentration of oestrogen in the incubation medium was measured directly without prior extraction, by RIA (Calogero *et al.* 1996). Aliquots (200 µl) of medium, diluted 1:100–1:200, or standard solutions were incubated with 100 µl anti-oestrogen serum (final dilution 1:10 000), and 100 µl [³H]17 β -oestradiol (about 10 000 c.p.m.) at 4 °C for 18–20 h. Ice-cold charcoal-dextran was then added to achieve separation of bound from free labelled hormone. Tubes were centrifuged at 1500 g at 4 °C for 15 min, and the supernatants were collected and counted in a β -counter after the addition of scintillation liquid. Total and nonspecific binding values were $33.6 \pm 2.1\%$ and $2.1 \pm 0.1\%$ respectively. Intra- and inter-assay coefficients of variation were $4.5 \pm 0.6\%$ and 9.3% respectively.

IGF-I and IGFBP-3 measurements

The media conditioned by rat granulosa cells were acidified by 18 h dialysis at 4 °C against 1 mol/l acetic acid, lyophilised and resuspended with 0.1 mol/l acetic

acid-0.15 mol/l NaCl (pH of the mixture <3). IGF-I was separated from IGFBP by gel filtration on a Sephadex G-50 column (1.6 × 90 cm) equilibrated with 0.1 mol/l acetic acid-0.15 mol/l NaCl, pH 2.75. Fractions corresponding to 0–0.20 (corresponding to the IGFBP elution volume), 0.21–0.40, 0.41–0.70 (corresponding to the free IGF elution volume) and 0.71–1 partition coefficient between the liquid phase and the gel phase (K_{av}) were pooled, lyophilised, reconstituted in PBS and analysed for IGF-I and IGFBP-3 immunoreactivity.

The IGF-I contents of media were evaluated in the fractions eluted from the Sephadex G-50 column in the molecular weight range of the free peptide. IGF-I was measured by double-antibody RIA using immunochemicals and tracer provided by Medgenix (Fleurus, Belgium). The intra-assay and interassay coefficients of variation were 6 and 7.5% respectively.

The IGFBP-3 contents of conditioned media were evaluated in the fractions eluted from the Sephadex G-50 column in the molecular mass range of more than 30 kDa. IGFBP-3 was measured by immunoassay using reagents and tracer provided by Bioclone Australia Pty Ltd (Narrrickville, NSW, Australia). The intra-assay and interassay coefficients of variation were 4.25 and 6.6% respectively.

Data analysis

Results, reported as means ± S.E.M. throughout the study, are expressed as percentage of basal hormone production. Statistical evaluation was performed by one-way analysis of variance (ANOVA) followed by the Duncan's Multiple Range test. Correlation analysis was performed using Pearson's correlation test. Significance was accepted at a *P* value lower than 0.05.

Results

Effects of CRH on oestrogen, IGF-I, and IGFBP-3 release

In basal conditions, granulosa cells released 1.26 ± 0.03 ng/well oestrogens, 7.92 ± 1.47 ng/flask IGF-I and 3.61 ± 0.47 ng/flask IGFBP-3. CRH at a concentration of 10^{-7} mol/l suppressed FSH (1 IU/l)-stimulated oestrogen (Fig. 1, upper panel) and IGF-I (Fig. 1, lower panel) release, whereas it had no effects on the release of either hormone in basal conditions or following exposure to 5 IU/l FSH. The amount of IGF-I released into the incubation medium correlated significantly with that of oestrogen ($r=0.30$, $P=0.02$, $n=58$). To evaluate whether the suppressive effects of CRH were receptor-mediated, we incubated granulosa cells with a tenfold excess of the CRH receptor antagonist, α -helical CRH₉₋₄₁ which overcame the effects of CRH on both oestrogen and IGF-I release (Fig. 2). Since the antagonist alone did not have any

Figure 1 Effects of CRH on oestrogen (upper panel) and IGF-I (lower panel) release by rat granulosa cells under basal conditions and following administration of 1 or 5 mIU/ml FSH. CRH suppressed oestrogen and IGF-I release. Results are expressed as means ± S.E.M.

effect on oestrogen or IGF-I release (data not shown), these data suggest that the suppressive effects of CRH on oestrogen and IGF-I release were receptor-mediated. CRH did not have any effect on IGFBP-3 release (data not shown).

Effect of CRH on oestrogen release stimulated by IGF-I

A preliminary dose-response study showed that IGF-I (25, 50 and 100 ng/ml) stimulated oestrogen release in a concentration-dependent manner. At the highest concentration tested, IGF-I was as effective as 1 IU/l FSH (data not shown). We then tested the effects of CRH on the oestrogen release stimulated by these three concentrations of IGF-I. CRH at concentrations of 10^{-8} or 10^{-7} mol/l did not have any effect on IGF-I-stimulated oestrogen release (Fig. 3). The addition of low concentrations of IGF-I (25 or 50 ng/ml) was, on the other hand, able to overcome the suppressive effects of CRH on FSH (1 IU/l)-stimulated oestrogen release (Fig. 4), suggesting

Figure 2 Effects of the CRH receptor antagonist α -helical CRH₁₋₄₁ (ANT) on FSH (1 IU/l)-stimulated oestrogen (upper panel) and IGF-I (lower panel) release. The suppressive effects of CRH on oestrogen and IGF-I release were receptor-mediated. Results are expressed as means $\pm$ S.E.M. Basal=experiments without CRH and ANT.

Figure 4 Effects of 25 ng/ml (upper panel) or 50 ng/ml (lower panel) IGF-I on the suppressive effects of CRH on FSH (1 IU/l)-stimulated oestrogen release. The exogenous addition of IGF-I overcame the suppressive effects of CRH. Results are expressed as means $\pm$ S.E.M. Basal=experiments without FSH, CRH and IGF-I; Control=experiments with FSH 1 IU/l alone.

Figure 3 Effects of increasing concentrations of CRH on oestrogen release stimulated by increasing concentrations of IGF-I. CRH did not have any effect on IGF-I-stimulated oestrogen release. Results are expressed as means $\pm$ S.E.M. Basal=experiments without IGF-I and CRH. * P <0.05 compared with respective basal value.

that the suppressive effect of CRH on oestrogen release may relate to the inhibition of endogenous IGF-I release.

Discussion

Immunoreactive CRH and CRH receptors have been found in rat ovaries (Mastorakos *et al.* 1993). Ovarian CRH is produced locally since both mouse and human ovarian tissue express CRH mRNA (Muglia *et al.* 1994, Asakura *et al.* 1997). By specific *in situ* hybridisation, Nappi and Rivest (1995) confirmed the presence of CRH receptors in the ovary and characterised them as type 1. These findings suggest that CRH may play an autocrine/paracrine role on ovarian function. Indeed, the studies undertaken to evaluate the role, if any, of CRH on rat ovarian function have shown that the neuropeptide inhibits ovarian steroidogenesis. Preliminary studies showed that CRH suppresses the production of androsterone from ovarian cell dispersates and of progesterone from granulosa cells luteinized by pretreatment with FSH (Mastorakos

et al. 1992). We have shown that CRH at elevated concentrations (i.e. 10^{-8} and 10^{-7} mol/l) inhibits FSH-stimulated oestrogen production by rat granulosa cells, whereas at a lower concentration (i.e. 10^{-10} mol/l), it causes a rightward shift of the oestrogen dose-response curve to FSH (Calogero *et al.* 1996). The capability of CRH to suppress ovarian steroidogenesis has also been confirmed in human granulosa-luteal cells (Calogero *et al.* 1996, Ghizzoni *et al.* 1997). The mechanism(s) of this inhibition is, however, unclear. We found that CRH suppresses the activity of the enzyme aromatase from rat granulosa cells without affecting their intracellular content of cAMP, the second messenger of FSH (Catt & Pierce 1986). Hence, it is not clear whether CRH acts directly on aromatase expression and/or function or whether it modulates other cAMP-independent cellular effects.

Because of the well-known stimulatory role of IGF-I on oestrogen release and aromatase activity in granulosa cells (Adashi *et al.* 1985a), we addressed the question whether CRH suppresses oestrogen release by interfering with the production of IGF-I from rat granulosa cells. The results of this study show that CRH is capable of inhibiting the production of IGF-I. The suppression of IGF-I release in the medium of incubation was completely abolished by the addition of a tenfold excess of the CRH receptor antagonist α -helical CRH₉₋₄₁, indicating that this effect was receptor-mediated. Thus, the effects of CRH on oestrogen production could be mediated, at least in part, by inhibiting the autocrine/paracrine action of IGF-I. This is further supported by the lack of CRH effect on oestrogen release stimulated by exogenously added IGF-I and by the finding that CRH-induced suppression of FSH-stimulated oestrogen release was overcome by low concentrations of exogenously added IGF-I. IGFBP-3 is a protein which binds specifically IGF-I and with this mechanism it interferes with the effects of IGF-I. Indeed, the addition of IGFBP-3 to granulosa cell cultures inhibited the production of oestrogen in response to FSH (Ui *et al.* 1989). The lack of CRH effect on IGFBP-3 production suggested that this peptide acts on the ovarian IGF system in a rather selective manner.

In conclusion, CRH suppressed the production of IGF-I, but not of IGFBP-3, by rat granulosa cells in a CRH receptor-mediated fashion. This suggested that the suppressive effects of CRH on ovarian oestrogen release may, at least under these experimental conditions, be mediated by a decreased release of IGF-I. These data add further circumstantial evidence for a wider role of CRH on ovarian function since IGF-I modulates other ovarian functions.

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