Endocrine (1995) 3, 395-398

Estrogens decrease expression of the corticotropin-releasing factor gene in the hypothalamic paraventricular nucleus and of the proopiomelanocortin gene in the anterior pituitary of ovariectomized rats

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It is known that estrogens modulate the hypothalamo-pituitary-adrenal (HPA) axis both under resting conditions and during exposure to stress. Nevertheless, the site of action of estrogens is not still fully elucidated. We sought to determine if estrogens could act on the major hypothalamic ACTH secretagogue: corticotropin-releasing factor (CRF). Mature rats were ovariectomized (OVX) and 2 weeks later implanted with silastic capsules containing 17β-estradiol (E_2). Animals were sacrificed 7 days later. CRF mRNA in the hypothalamic paraventricular nucleus (PVN) and proopiomelanocortin (POMC) mRNA in the anterior pituitary were measured by *in situ* hybridization. CRF content in the median eminence was measured by semi-quantitative immunocytochemistry. E_2 treatment induced a significant decrease of CRF mRNA levels in the PVN (3.70 ± 0.14 *vs* 4.79 ± 0.15 copies of probe $\times 10^{-3}/\mu m^3$ of tissue in OVX rats, $P < 0.05$), an accumulation of immunoreactive CRF in the zona externa of the median eminence (207 ± 36 *vs* $100 \pm 15\%$ in OVX rats, $P < 0.05$), and a decrease of POMC mRNA levels in the anterior pituitary (4.6 ± 0.6 *vs* 6.9 ± 0.6 copies of probe $\times 10^{-2}/\mu m^3$ of tissue in OVX rats, $P < 0.05$). These results demonstrate that estrogens have a negative effect on CRF gene expression and secretion and on POMC gene expression. Whether estrogens modulate directly the CRF-synthesizing cells or act through an increase of the glucocorticoid negative feedback remains to be determined.

Keywords: Estrogens; corticotropin-releasing factor; proopiomelanocortin; paraventricular nucleus; anterior pituitary; *in situ* hybridization

Introduction

Complex interactions exist between the hypothalamo-pituitary-adrenal (HPA) and hypothalamo-pituitary-gonadal axis. It is established that corticotropin-releasing factor (CRF), one of the major hypothalamic regulators of ACTH and corticosterone secretion, stimulates hypothalamic opioid peptides which inhibit the release of gonadotropin-releasing hormone from the hypothalamus. This phenomenon explains the disruptive effects of stress on reproductive function that has been observed in several species including humans (for review see Rivier & Rivest, 1991). Gonadal steroids, in turn, are known to affect the HPA axis under both basal and stress conditions. Female rats have enhanced basal corticosterone levels and stress-induced ACTH and corticosterone secretion (Critchlow *et al.*, 1963; Viau & Meaney, 1991). These changes in the stress regulation of the HPA axis are abolished by ovariectomy (OVX) and reversed by estrogen administration (Le Mevel *et al.*, 1978; Burgess & Handa, 1992). The site of action of estrogens in modulating ACTH and corticosterone secretion is not fully elucidated. Estrogens may have a direct effect at the adrenal level, by stimulating adrenal production of corticosterone (Kitay, 1963). Alternatively, estrogens could act at the level of the anterior pituitary. ACTH secretion stimulated by hypothalamic extracts is decreased in OVX rats, this effect being reversed after estrogen treatment (Coyne & Kitay, 1969). Finally, estrogens may act at the hypothalamic level to regulate CRF biosynthesis. Indeed, chronic treatment with estrogens decreases hypothalamic immunoreactive CRF (Haas & George, 1989) while it has been demonstrated that CRF mRNA increases in the afternoon of proestrus in the parvocellular portion of the hypothalamic paraventricular nucleus (PVN; Bohler *et al.*, 1990). In this report we searched for a possible modulation of CRF gene expression after estrogen treatment of OVX rats. CRF mRNA levels in the hypothalamic PVN and proopiomelanocortin (POMC) mRNA concentrations in the anterior pituitary were measured using *in situ* hybridization. CRF immunoreactivity was measured in the median eminence using semi-quantitative immunocytochemistry.

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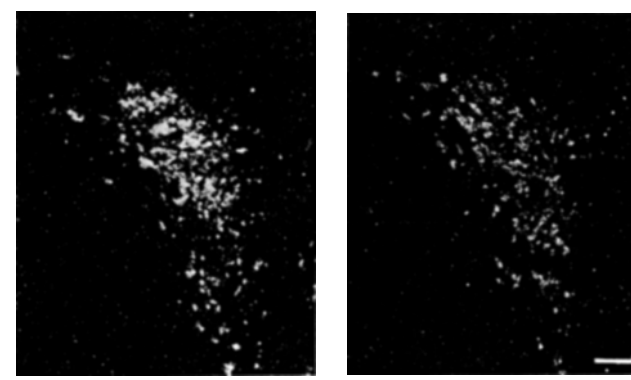
Received 20 September 1994; accepted 21 February 1995

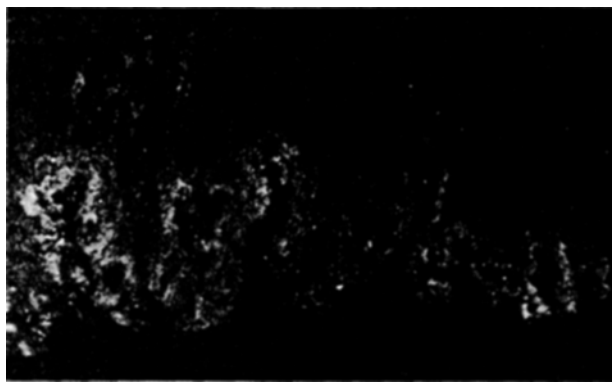
Results

In OVX rats, a clear hybridization signal for CRF was recognized in the medial parvocellular part of the PVN (Figure 1). Quantitative analysis of the film autoradiograms showed that the intensity of the hybridization signal for CRF was reduced in OVX-E_2 animals (3.70 ± 0.14 *vs* 4.79 ± 0.15 copies of probe $\times 10^{-3}/\mu m^3$ of tissue in OVX rats, $P < 0.05$). Figure 2 shows the result of immunostaining for CRF. E_2 treatment induced a significant accumulation of immunoreactive CRF in the zona externa of the median eminence

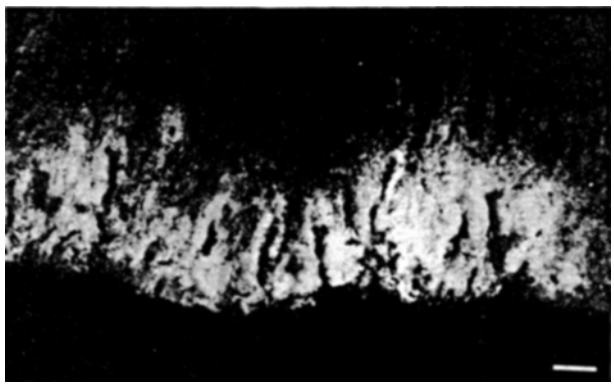


OVX OVX-E_2

Figure 1 Darkfield view of the effect of E_2 treatment on CRF gene expression in the hypothalamic PVN. Brain sections obtained from OVX or OVX-E_2 rats were hybridized as described in Materials and methods. Bar equals 100 μm

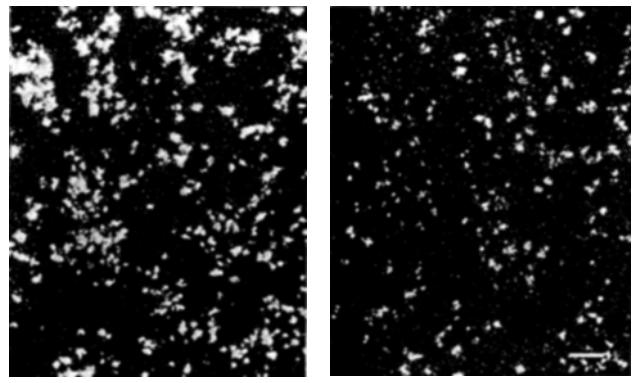


OVX



OVX-E_2

Figure 2 Effect of E_2 treatment on CRF immunoreactivity in the zona externa of the median eminence. Brain sections obtained from OVX or OVX-E_2 rats were immunocytochemically stained for CRF as described in Materials and methods and photographed under fluorescence microscopy. Bar equals 20 μm



OVX OVX-E_2

Figure 3 Darkfield view of the effect of E_2 treatment on POMC gene expression in the anterior pituitary. Pituitary sections obtained from OVX or OVX-E_2 rats were hybridized as described in Materials and methods. Bar equals 100 μm

shows the result of the hybridization for POMC in the anterior pituitary. E_2 treatment resulted in a significant ($P<0.05$) decrease of the POMC hybridization signal (4.6 ± 0.6 *vs* 6.9 ± 0.6 copies of probe $\times 10^{-2}/\mu m^3$ of tissue in OVX rats).

Discussion

Our results demonstrate that estrogens have a negative effect on CRF gene expression in the PVN. The decrement of CRF mRNA levels was accompanied by an accumulation of immunoreactive CRF in the zona externa of the median eminence, suggesting that E_2 administration decreases both CRF synthesis and secretion. This observation is in contradiction with the recent report of Redei *et al.* (1994) who showed that E_2 treatment of OVX rats had no effect on CRF mRNA levels in extracts of hypothalamic tissue blocks extending from the optic chiasm to the mammilary bodies and from the median eminence to the anterior commissure. Nevertheless, such measurements include CRF mRNA present in areas other than the parvocellular PVN, such as the bed nucleus of the stria terminalis, the medial preoptic area, the substancia innominata, the caudal periventricular region, and the dorsomedial and lateral hypothalamic areas (Swanson *et al.*, 1983; Kawano *et al.*, 1988). It is conceivable that moderate changes of CRF gene expression in the parvocellular PVN could not be detected by measuring CRF mRNA levels in whole hypothalamic tissue blocks. At the level of the anterior pituitary, E_2 treatment induced a decrease of POMC mRNA levels. This observation is consistent with the data of Redei *et al.* (1994). It is reasonable to hypothesize that the alteration of ACTH biosynthesis in E_2-treated rats is, at least partly, consecutive to the decreased CRF synthesis and secretion since it is established that CRF has a stimulatory effect on POMC gene transcription (Gagner & Drouin, 1985). Alternatively, estrogens could act directly at the level of the anterior pituitary (Coyne & Kitay, 1969) since estrogen receptors mRNA has been identified in corticotrope cells of normal human pituitaries (Stefaneanu *et al.*, 1994).

The site(s) of action and the mechanism(s) by which estrogens modulate CRF gene expression are not clear. Estrogens could act directly at the level of the PVN since estrogen binding is present on some parvocellular neurons of the rat PVN (Rhodes *et al.*, 1981). Haas & George (1989) have shown that E_2 decreases the concentrations of immunoreactive CRF in the median eminence, this effect being consecutive to a decreased CRF synthesis. The discrepancy between the decrease of CRF immunoreactivity after E_2 treatment found by Haas & George after and the increase found in the present study could be explained by the longer treatment period (three weeks) used in the former study. Indeed, the initial build-up of CRF immunoreactivity observed after one week of E2 treatment could subsequently dissipate. It has been reported that the human CRF gene contains in its promoter region estrogens responsive elements and that estrogens induce increased transcription of the CRF gene when studied *in vitro* (Vamvakopoulos & Chrousos, 1993). In addition, CRF mRNA levels are elevated on the afternoon of proestrus, a time at which there is a surge of estrogens secretion (Bohler *et al.*, 1990). These observations are in apparent discrepancy with our present data. Regarding the former study, it is possible that the human and the rat CRF genes present structural differencies or are regulated in an opposite manner by the same hormone. For example, it known that, in human placenta, glucocorticoids have a positive effect on CRF gene transcription (Robinson *et al.*, 1988), while in rats it is established that glucocorticoids have a negative effect on CRF gene expression in the hypothalamus (Young *et al.*, 1986). In addition, the above mentioned results were obtained *in vitro* and therefore do not take in account possible influences of neural and/or humoral inputs to the CRF-synthesizing cells in response to estrogens removal or exposure. Regarding the variations of CRF gene expression during the estrous cycle, the concomitant existence of an estrogen surge and an increase of CRF gene expression does not necessarily imply that estrogens are responsible for the modulation of CRF gene synthesis. In addition, the changes of CRF mRNA levels during the afternoon of proestrus have been observed in a limited portion of the PVN, namely the ventral portion of the parvocellular zone (Bohler

et al., 1990). This is in opposition with our data which show changes of CRF mRNA levels in the total parvocellular portion of the PVN. In addition, under our experimental conditions, rats were exposed to circulating concentrations of E_2 in the range of 40 pg/ml (Gogan *et al.*, 1980), i.e. values comparable to those reached during the afternoon of proestrus (Legand *et al.*, 1975), but for a prolonged period of time (7 days). Another hypothesis that could explain the modulation of CRF gene expression by E_2 is an indirect effect of estrogens. It is established that E_2 treatment of OVX rats induces an increase of basal plasma corticosterone concentrations (Critchlow *et al.*, 1963). This effect could be consecutive either to an increased sensitivity of the anterior pituitary to the stimulatory effect of CRF and/or AVP (Coyne & Kitay, 1969) or to a direct effect of estrogens at the adrenal level (Kitay, 1963). In addition, it is known that E_2 treatment increases both glucocorticoid receptor binding and glucocorticoid receptor mRNA in the hypothalamus (Ferrini & De Nicola, 1991; Redei *et al.*, 1994). These observations suggest that estrogens could increase the negative glucocorticoid feedback which in turn could modulate CRF and POMC gene expression, since it is established that glucocorticoids down regulate CRF gene transcription in the PVN (Young *et al.*, 1986) and POMC gene transcription in the anterior pituitary (Gagner & Drouin, 1985). Further experiments, investigating the effect of removal of circulating glucocorticoids and/or estrogens will be helpful in understanding the regulation by estrogens of the hypothalamo-pituitary-adrenal axis.

Materials and methods

Animals

Female Sprague-Dawley rats (180-200 g BW) were purchased from the Centre d'Elevage R. Janvier (le Genest St. Isle, France). They were housed in our laboratory under controlled temperature (22-24°C) and a constant 12 h light/dark cycle for at least 1 week before the experiment. They had free access to standard rat chow and tap water. Bilateral ovariectomy ($n = 12$) was performed under ether anesthesia using a dorsal approach. Two weeks later six rats were implanted with 17β-estradiol (E_2) contained in a 0.5 cm silastic capsule inserted under the skin (Gogan *et al.*, 1980) under light ether anesthesia. The two groups of rats were sacrificed by decapitation 1 week later. Brains and pituitaries were carefully removed, immediately frozen on dry ice, and stored at −70°C until sectioning.

In situ *hybridization*

Sixteen consecutive coronal sections (12 μm through the hypothalamic PVN between levels A 5660 μ and A 5340 μ of the atlas of König & Klippel (1967) were cut in a cryostat microtome at −20°C. The sections were thaw-mounted onto twice gelatin-coated slides, dried on a slide warmer and kept at −70°C. They were divided in four groups of four consecutive sections. The first sections from each of the four groups were hybridized with the CRF probe. Four consecutive coronal sections were cut through the median eminence (between levels A 4230 μ and A 4110 μ), and four consecutive horizontal sections were cut through the pituitary (approximately at a middle level, i.e. when the three lobes of the pituitary are clearly visible and when the intermediate lobe shows the typical horseshoe shape). *In situ* hybridization was performed as previously described (Young *et al.*, 1986) with slight modifications. The sections were warmed at room temperature and fixed with 4% formaldehyde in PBS, pH 7.2, for 5 min, After two washes in PBS, they were placed in 0.25% acetic anhydride in 0.1 M triethanolamine-0.9% NaCl, pH 8.0, for 10 min and delipidated in ethanol and chloroform. They were prehybridized at 37°C with unlabelled deoxyadenosine-α-thiotriphosphate (50 μM, New England Nuclear, Paris, France) in a buffer containing 4 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.2), 50% (vol/vol) formamide, 10% (wt/vol) dextran sulphate, 50 μg/ml sheared single-stranded salmon sperm DNA, 25 μg/ml yeast tRNA, 1 × Denhardt's solution, 0.1 M dithiothreitol under a parafilm coverslip. After 2 h the coverslip and as much buffer as possible were carefully removed and 40 (brain sections) or 10 μl (pituitary sections) of the same buffer containing 1×10^6 d.p.m. of CRF probe (brain sections) or 0.25×10^6 d.p.m. of POMC probe (pituitary sections) were added. After a 20 h incubation at 37°C, the sections were washed in four 15 min rinses of 2 × SSC containing 50% formamide at 40°C, followed by two 30 min rinses of 1 × SSC at room temperature. The slides were exposed to X-ray films (X-OMAT AR5, Kodak, Le Pontet, France), concomitantly with radioactive brain paste standards, for 12 h (POMC hybridization) or two weeks (CRF hybridization) and then dipped in nuclear emulsion (1:1 in water, K5, Ilford, Saint-Priest, France) and exposed for 2 days (POMC hybridization) or 4 weeks (CRF hybridization).

Synthetic oligonucleotides directed against bases 496-543 of the rat CRF mRNA (Jingami *et al.*, 1985) or bases 349-396 of the rat POMC mRNA (Drouin *et al.*, 1985) were used as probes. The probes were labelled using [^{35}S]deoxyadenosine thiotriphosphate (1300 Ci/mmol, New England Nuclear) and terminal deoxynucleotidyl transferase (Boehringer Mannheim, Meylan, France). The specific activity of the radioactive probes was about 9000 or 12 000 Ci/mmol for the CRF and POMC probes, respectively. The specificity of the CRF probe has already been described (Grino *et al.*, 1990). The specificity of the POMC probe was assessed by Northern blot hybridization of rat anterior extracts. Under this condition the POMC probe hybridized with a single mRNA species of about 1 kb (data not shown).

Quantification of the hybridization signal

All the sections that were hybridized were analysed. The hybridization signal was quantified on the film autoradiograms. All the images were digitized during the same session. The optical density of the hybridized areas was measured using a Biocom 200 (Biocom, Les Ulis, France) image analysis system. Optical densities were converted to copies of probe per μm^3 of tissue, using the radioactive brain paste standards, according to Young *et al.* (1986).

Immunohistochemistry

The sections were warmed at room temperature and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 15 min. After three washes in PB, they were incubated for 30 min in PB containing 3% normal sheep serum (NSS) and 0.1% Triton X_{100}. Sections were incubated overnight at room temperature in PB containing 1% NSS, 0.1% Triton X_{100} (PB/NSS/TX) and the antiserum anti-CRF (CRF-AS) diluted 1/1000. The sections were washed 5 min three times in PB/NSS/TX, further incubated for 3 h in PB/NSS/TX containing the second antibody (fluorescein-conjugated goat anti-rabbit IgG, Jackson Immunoresearch Laboratories, distributed by Immunotech, Marseille, France) diluted 1/200 and subsequently washed 10 min three times in PB. After coversliping with mowiol (25% (wt/vol) in 25% glycerol, 0.1 M Tris pH 8.5), the sections were examined and photographed (Agfapan, 400 ASA) using a Zeiss Universal microscope equipped with a 50-W mercury-arc lamp and an appropriate filter set for FITC with a ×25 objective. The CRF-AS (rCRF 3) was raised in a rabbit using synthetic rat CRF (Peninsula, Meyerside, UK) coupled to BSA with carbodiimide. The CRF-AS showed no cross-reaction with the following peptides (diluted at a concentration of 1 μg/ml in

the CRF/AS incubation buffer): AVP, substance P, thyrotropin-stimulating hormone, growth hormone-releasing hormone, neuropeptide Y, neurotensin, luteinizing hormone-releasing hormone, somatostatin, vasoactive intestinal peptide and leucine- and methionine-enkephalin. Omission of the CRF-AS or of the second antibody resulted in a lack of signal. Adding increasing doses (ranging from 1 to 10 ng/ml) of synthetic rat CRF to the CRF-AS incubation buffer resulted in a dose-dependent ($r^2 = 0.918$, $P = 0.01$) linear decrease of the optical density of the signal. Quantitative analysis was performed on the film negatives. All the images were digitized during the same session. The integrated optical density was measured in the half external part (i.e. the zona externa) of the median eminence using the image analysis system. The mean area from which the measurements were taken was comparable between the two groups (326 ± 17 and $352 \pm 24\ \mu m^2$ in OVX and OVX-E_2, respectively, $P > 0.05$). Since the total scale of optical density (0–255) of the image analysis system was wider than the real scale of the immunocytochemistry signal, optical densities were converted in arbitrary units using the standard curve.

Statistical analysis

All data are presented as the mean ± SE. Statistical analysis was performed using the unpaired two-tailed Student's *t* test for *in situ* hybridization data or the Mann-Whitney *U* test for the immunocytochemical data.

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