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ESTRADIOL REGULATES mRNAs ENCODING PRECURSORS TO RAT LUTROPIN (LH) AND FOLLITROPIN (FSH) SUBUNITS

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SUMMARY. Anterior pituitary mRNA was prepared using a microscaled method and translated in a wheat-germ cell-free system in the presence of [^{35}S] labeled cysteine and methionine. Translation products, immunologically related to LHβ, FSHβ and the common subunit α, were isolated as precursors with antisera to denatured subunits and characterized by SDS-polyacrylamide gel electrophoresis and fluorography. The radioactive bands were excised from the gel and counted for quantitative evaluation. Our data show that translation of pituitary mRNAs from ovariectomized (ovx) rats results in precursor levels increased by 10 fold for α and 14 fold for LHβ as compared to the levels in normal rats. The increase in FSHβ precursor was impossible to evaluate as a specific immunoprecipitation product was undetectable in the case of normal rats. Estradiol, but not progesterone, administered *in vivo* to ovariectomized rats, reversed the stimulatory effect of ovariectomy on the expression of mRNAs coding for gonadotropin subunit precursors 48 h after injection. These results suggest that estradiol, but not progesterone, negatively regulates the synthesis of the pituitary gonadotropins in the female rats via changes in specific mRNA levels. This hormonal control probably occurs at the transcriptional level.

The gonadotropins, lutropin (LH) and follitropin (FSH), are both synthesized and secreted by gonadotropic cells of the anterior pituitary, as shown by immunohistochemical studies (1). By their regulatory action on gonads, gonadotropins play a very important role in the process of reproduction. Like another pituitary glycoprotein hormone, thyrotropin (TSH), LH and FSH are composed of two dissimilar, noncovalently linked α- and β-subunits. Within a species, the aminoacid sequence of the α-subunit of all glycoprotein hormones, LH, FSH and TSH, and human chorionic gonadotropin (hCG), is identical; only the β-subunits, which confer to a hormone its biological and immunological specificity, differ (reviewed in 2).

The secretion of LH and FSH is controlled by a number of factors

Abbreviations: LH, lutropin; FSH, follitropin; TSH, thyrotropin; GnRH, gonadoliberin; ovx, ovariectomized; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RCXM, reduced and carboxymethylated (subunits); E_2, 17β-estradiol; P, progesterone.

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including mainly gonadoliberin (GnRH, or gonadotropin-releasing hormone) from the hypothalamus, and the gonadal steroids (3). Although a considerable amount of work on the cellular mechanisms of action of GnRH and gonadal steroids in the secretion of gonadotropins has been accomplished, to our knowledge, very little progress has been made toward understanding the hormonal control of LH and FSH biosynthesis. It has recently been reported that orchidectomy of male sheep (4) and ovariectomy of female rats (5,6) dramatically enhance the levels of specific mRNAs encoding LHα- and β-subunits (5) and FSHβ (4,5) as reflected by their cell-free translation. Administration of 17β-estradiol (E_2) to castrated animals reverses the effect of gonadectomy and brings the level of gonadotropin subunits near that present in normal animals (4,7).

As a development of our preliminary study (5,7), we report here further details of the regulation of mRNAs encoding rat LH and FSH subunits by E_2. Specific mRNAs activity was measured by cell-free translation of pituitary RNA in a wheat-germ system, followed by specific immunoprecipitation of the subunit precursors and their characterization using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

MATERIALS AND METHODS

Reagents : The following reagents were purchased: [^{35}S]-labeled cysteine and methionine (spec. act. ≃ 1000 Ci/mmol): NEN-France; nucleotides: Boehringer (Mannheim, FRG); micrococcal nuclease: Worthington; cesium chloride (suprapur): Merck (Darmstadt, FRG); oligo(dT)-cellulose: Collab. Res. (Waltham, MA); sarkosyl: Ciba-Geigy (Basle, Switzerland); X-ray film (RP royal X-AR): Kodak. All other reagents used were A-grade.

Preparation and translation of rat pituitary mRNA : Three month old Wistar female rats (laboratory strain) were randomized and divided into 5 groups. Group 1 served as a normal control and the remaining 4 groups were ovariectomized (ovx). Four weeks later, groups 1 and 2 (ovx control) were injected with oil and the remaining 3 groups were injected with following steroids: group 3, 25 μg E_2; group 4, 12.5 mg progesterone (P); group 5, 25 μg E_2 + 12.5 mg P. Fourty eight hours later, rats were sacrificed, anterior pituitary glands were excised, quick-frozen and stored in liquid nitrogen. Due to the small number of pituitaries in each group (7/group, equivalent to 45-50 mg fresh tissue), we have used a micromethod for extraction of total RNA. This method (8) involved homogenization of 5-10 rat pituitaries in 2.3 ml of a buffer containing 4 M guanidinine-HCl, 1 % sarkosyl, 0.1 M 2-mercaptoethanol, 50 mM sodium acetate, pH 5. The homogenate was layered on a cushion of 3.1 ml 5.7 M CsCl-0.1 M EDTA (9) and centrifuged at 33000 rpm for 15 h at 20°C in a "SW 50.1" rotor (Beckman). The pellet was dissolved in 300 μl 0.1 M sodium acetate pH 7.0-0.5% SDS and reprecipitated twice with ethanol. For the second precipitation no SDS was added. Before

translation, RNA was heat-denatured (5 min at 60°C) and its messenger activity was tested by measuring the incorporation of [^{35}S]methionine into total protein as a function of RNA concentration (6).

Translation was achieved in optimized conditions in the presence of [^{35}S]methionine and [^{35}S]cysteine (0.6 mCi/ml of each), using a wheat-germ extract deprived of endogenous messenger activity by a pretreatment with micrococcal nuclease (10). Labeled polypeptides were isolated from the translation media by immunoprecipitation with specific antisera to reduced and carboxymethylated (RCXM) bovine LHα, provided by Dr John Pierce, and ovine LHβ and FSHβ, raised in rabbits in our laboratory. Immunoprecipitation was achieved as previously described (10,11) with the translation products subjected to reduction and alkylation. Immunoprecipitated, labeled polypeptides were characterized by electrophoresis on SDS-17 % polyacrylamide slab gels and revealed by fluorography. For quantitative determination, the radioactive bands were excised from the gel, solubilized with H_2O_2 and counted; cpm were corrected for quenching and isotope decay (11).

RESULTS

We first verified that the messenger activity of mRNA preparations from either group in the same experimental series was the same or nearly the same (data not shown). The method, standardized as described, was highly reproducible in terms of maximal translational activity and RNA dose-dependence activity (8). The difference in the messenger responses between two RNA preparations never exceeded 10 %, and maximal peptide synthesis was obtained in 50 µl with RNA extracted from 0.8 hypophysis. Following translation in the presence of sub-maximal RNA concentration, identical aliquots of translation media were immunoprecipitated with specific antisera and submitted to SDS-PAGE and fluorography.

Fig. 1 shows a fluorograph of the [^{35}S]-labeled polypeptides immunoprecipitated with antiserum to RCXM-α from the translation media of mRNAs derived from normal female rats, ovx rats and ovx rats supplemented with E_2 and P or a combination of both. Though the translation of mRNAs from each origin was carried out in the same conditions, and the same amount of radioactivity was used for immunoprecipitation, it is evident that ovariectomy increased the amount of α precursor synthesized (lane 2) while treatment of rats *in vivo* with E_2 lowered this amount (lane 3). Treatment with P was apparently without effect (lane 4) and that with both E_2 + P gave about the same results as E_2 alone (lane 5).

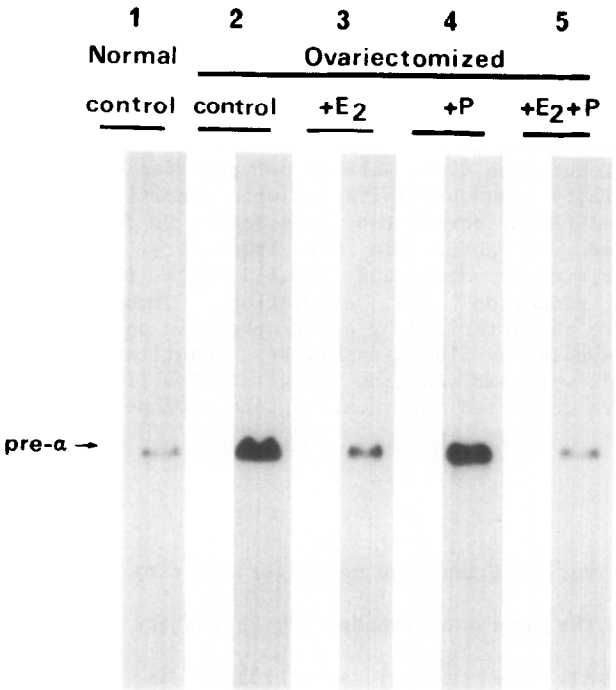

Fig. 1 : Fluorograph of [^{35}S]α precursor synthesized in a wheat-germ system in response to mRNA derived from normal female rats, ovariectomized (ovx) rats and ovx rats supplemented with 17β-estradiol (E_2) or progesterone (P), or a combination of both (E_2 + P). Rats were injected 4 weeks after ovariectomy (s.c.) with 25 μg E_2 and/or 12.5 mg P. They were sacrificed 48 h later and anterior pituitary mRNA was prepared and translated as indicated above. Subunit α precursor was immunoprecipitated with specific antiserum against denatured α and characterized by SDS-17% polyacrylamide slab gel electrophoresis and fluorography. Numbering of lanes: 1 control, normal female rat; 2, control, ovx rat; 3, ovx rat + E_2; 4, ovx rat + P; 5, ovx rat + E_2 + P.

Very similar results were obtained when [^{35}S]-labeled polypeptides were precipitated with antisera to RCXM-LHβ and RCXM-FSHβ. In this latter case, the band corresponding to FSHβ precursor was detectable only in the translation media derived from ovx rats and ovx rats treated with P (data not shown).

Table I shows a quantitative evaluation of the radioactivity incorporated into α, LHβ and FSHβ precursors isolated by SDS-PAGE in each group. For the α precursor, the radioactivity incorporated (as % of incorporation in total protein) increased from 0.21 % in normal rats to 2.0 % in ovx rats. Treatment with E_2 decreased this radioactivity to 0.46 %, P

TABLE 1. Influence of ovariectomy with or without treatment with ovarian steroids on the messenger activity of mRNA encoding gonadotropin subunits[a].

Source of mRNA	[^{35}S]Cys + [^{35}S]Met (cpm) incorporated into:			
	T P[b]	pre-α[c]	pre-LHβ[c]	pre-FSHβ[c]
1. Normal female rats	945000	2025 (0.21%)[e]	375 (0.04%)	und[d]
2. Ovariectomized rats (ovx)	960000	19200 (2.00%)	5340 (0.56%)	480 (0.05%)
3. ovx + E_2 (25μg)	970000	4450 (0.46%)	925 (0.09%)	und
4. ovx + P (12.5 mg)	956000	19380 (2.03%)	5225 (0.55%)	460 (0.05%)
5. ovx + E_2 (25 μg) + P (12.5 mg)	965000	4150 (0.43%)	950 (0.10%)	und

[a] RNA was extracted in parallel from anterior pituitaries of normal female rats, ovariectomized rats (ovx) and ovx rats treated with gonadal steroids (ovx + E_2, ovx + P, ovx + E_2 + P), then translated in cell-free conditions. Translation products were characterized and quantified according to the methodology described in the text .

[b] T P = total protein: polypeptides precipitated with 10 % TCA in 12 μl of translation media.

[c] Specific immunoprecipitates purified by SDS-polyacrylamide gel electrophoresis (see the text).

[d] und = undetectable.

[e] In parentheses: percent of radioactivity vs radioactivity incorporated into total protein (b).

E_2 = 17 β-estradiol; P = progesterone.

was without effect on incorporation and the combination of both, E_2 + P, gave results similar to those obtained with E_2 alone. Similarly, for the LHβ precursor, ovariectomy increased the incorporation of the radioactivity from 0.04 % to 0.56 % (14-fold increase) and treatment of ovariectomized rats with E_2 lowered this incorporation to 0.09 %. As previously, P was without effect, either alone or in the presence of E_2. On the other hand, ovariectomy brought the FSHβ precursor from undetectable values in normal rats to 0.05 % of the total incorporated radioactivity while P had no effect on incorporation.

Fig. 2 shows the incorporation of [^{35}S]methionine (expressed in cpm percent of translated proteins) into the α-precursor synthesized in response to rat pituitary mRNA prepared at different periods of time after ovariectomy. The proportion of the α-precursor increased as a function of time and reached a plateau about 3 weeks after ovariectomy.

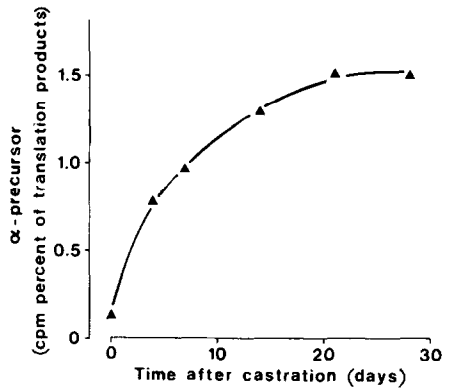

Fig. 2 : Incorporation of [^{35}S]Met (expressed in cpm percent of translated proteins) into the α-precursor synthesized in response to rat pituitary mRNA prepared at different periods of time after ovariectomy. Labeled α precursor was isolated by specific immunoprecipitation and SDS-polyacrylamide gel electrophoresis as indicated above.

DISCUSSION

It is well known that ovariectomy of the prepubertal and adult rats results in a dramatic rise in plasma LH (12, 13, 14). Similarly, in castrated rats of both sexes plasma FSH concentrations are 10-20 fold greater than the levels measured in intact male and diestrous female rats (15). Although it has been repeatedly established over more than 50 years that E_2 exerts a negative feedback on pituitary gonadotropin secretion (reviewed in 16), the mechanism of this inhibitory action as well as the site(s) of estrogen effect are still controversial. It has been shown that treatment of ovariectomized rats *in vivo* by E_2 + P consistently lowers plasma LH and FSH levels without appreciably altering pituitary gonadotropin stores (17). Using dispersed-cell cultures of the pituitary gland, Miller and Wu (18) reported that E_2 inhibited the spontaneous secretion of FSH in sheep, pig and cow cell culture and stimulated secretion of FSH in rat cell culture. These authors concluded that estrogens are capable of altering FSH secretion and synthesis differentially in pituitary cell cultures from different species. It has been shown more recently in the same laboratory that in castrated male sheep (4), serum FSH and pituitary FSHβ mRNA activity levels are reduced about 75 % after 3 days and 85-90 % after 6 days of E_2 treatment. Similarly, it

has been demonstrated that treatment of ovx ewes with E_2 + P significantly diminishes pituitary subunit α mRNA activity levels (19).

Using rats 4 weeks after ovariectomy as an experimental system and translation of extracted pituitary mRNA as methodology, we report here data showing that a single injection of 25 μg E_2, but not of P, into castrated animals, inhibited, 48 h after administration, the synthesis of all three gonadotropin subunit precursors, α, LHβ and FSHβ encoded in cell-free conditions by pituitary mRNAs. Inhibition of the mRNA encoding the α precursor was about 77 %, that of the LHβ precursor nearly 84 %. Because the FSHβ precursor was undetectable in the translation media of ovx rats treated with E_2 it was not possible to know the extent of inhibition of this subunit. Nevertheless, we shall assume it to be of the same order of magnitude as for other subunit precursors.

Thus, our results strongly suggest that, like ovine α and FSHβ (19,4), the synthesis of rat α, LHβ and FSHβ precursors is negatively controlled by E_2 and that specific mRNAs coding for all three subunits are regulated in a similar manner. At present we have no information on whether this control occurs by lowering the number of copies of the specific mRNAs coding for the subunit precursors or by decreasing the messenger activity of the specific mRNAs present. Another unsolved problem is that of the mechanism by which estradiol regulates those specific mRNAs. Many examples from the literature indicate that steroids may exert their regulatory role at the transcriptional level (reviewed in 20). However, in the majority of reported cases, E_2 acts by stimulating or inducing transcription. To our knowledge, inhibitory action of E_2 has been reported only in the case of the synthesis of ovine α (19) and FSHβ (4) subunits and extended to the rat subunits α, LHβ and FSHβ in this work. Since inhibition of the synthesis of ovine FSHβ by E_2 has been observed *in vitro* as well as *in vivo* (4), the pituitary gland can be considered, in this case, to be the direct target for estradiol action.

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REFERENCES

1. Tixier-Vidal, A., Tougard, C., Kerdelhué, B. and Jutisz, M. (1975) Ann. N.Y. Acad. Sci. 254, 433-461.
2. Pierce, J.G. and Parsons, T. (1981) Ann. Rev. Biochem. 50, 465-495.
3. Fink, G. and Pickering, A. (1980). Synthesis and Release of Adenohypophyseal Hormones (M. Jutisz and K.W. McKerns, eds.) Plenum Press, New York, pp. 617-638.
4. Alexander, D.C. and Miller, W.L. (1982) J. Biol. Chem. 257, 2282-2286.
5. Counis, R., Corbani, M. and Jutisz, M. (1982) Program of the 64th Annual Meeting of the Endocrine Society, abst. 195.
6. Counis, R., Corbani, M. and Jutisz, M. (1982) in "Pituitary Hormones and Related Peptides" (M. Motta, M. Zanisi and F. Piva, eds.) Academic Press, London, New York, pp.49-61.
7. Counis, R., Corbani, M. and Jutisz, M. (1983). In "Multihormonal Regulations in Neuroendocrine Cells", Proceedings of the INSERM European Symposium, Colmar (France) September 1-4, 1982, in press.
8. Counis, R., Corbani, M. Bérault, A., Théoleyre, M., Jansem de Almeida Cathano, M.T. and Jutisz, M. (1981) C. R. Acad. Sc. Paris, Series III, 293, 115-118.
9. Glisin, V., Crkvenjakov, R. and Byus, C. (1974) Biochemistry 13, 2633-2637.
10. Counis, R., Ribot, G., Corbani, M., Poissonnier, M. and Jutisz, M. (1981) FEBS Letters 123, 151-155.
11. Counis, R., Corbani, M., Poissonnier, M. and Jutisz, M. (1982) Biochem. Biophys. Res. Commun. 107, 998-1005.
12. Bodganove, E.M., Parlow, A.F., Bogdanove, J.N., Bhargava, I. and Crabill, E.V. (1964) Endocrinology 74, 114-119.
13. McCann, S.M. and Ramirez, V.D. (1964) Rec. Progr. Hormone Res. 20, 131-181.
14. Butterstein, G.M., Friedman, W., Rehder, M.C. and Wilion, F. (1983) Proc. Soc. Exp. Biol. Med. 172, 11-15.
15. Gay, V.L. and Sheth, N.A. (1972) Endocrinology 90, 158-162.
16. Schally, A.V., Kastin, A.J. and Arimura, A. (1972) Vitamins and Hormones 30, 83-164.
17. Gay, V.L. and Bogdanove, E.M. (1969) Endocrinology 84, 1132-1142.
18. Miller, W.L. and Wu, J. (1981) Endocrinology 108, 673-679.
19. Landefeld, T.D., Kepa, J. and Karsch, F.J. (1983) J. Biol. Chem. 258, 2390-2393.
20. Woo, S.L.C. and O'Malley, B.W. (1975) Life Sciences 17, 1039-1048.