Effect of clomiphene citrate on the in vitro release of LH and FSH by the pituitary gland of the long-term ovariectomized rat pretreated with LRH or with LRH and oestradiol benzoate

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Abstract. The effect of a combined in vivo pre-treatment with luteinizing hormone-releasing hormone (LRH) and either oestradiol benzoate (OB), clomiphene (-citrate) or OB plus clomiphene on the autonomous and the supramaximally LRH-stimulated in vitro secretion of LH and FSH by pituitary glands of long-term ovariectomized (OVX) rats was studied using a hemi-pituitary perfusion system. The concentration of LRH in the perfusion medium was 1 µg/ml. Pre-treatment with LRH during 5 days was effected by means of sc implanted Alzet® osmotic minipumps; control rats received a piece of silastic with the dimensions of a minipump. OB, 3 µg/injection, clomiphene 100 µg/injection or solvent were given on days 2 and 4 (day of perfusion: day 5). In rats not pre-treated with LRH neither OB, nor clomiphene changed the content of the pituitary gonadotropin stores. There was only a small but significant positive effect of the combined treatment with OB and clomiphene on the pituitary FSH content. LRH (partly) depleted the gonadotropin stores. This effect of LRH was potentiated by OB, but not by clomiphene. Clomiphene prevented the depleting-potentiating effect of OB. OB raised the LRH-stimulated secretion of LH and FSH as well as the autonomous secretion of LH. Clomiphene raised the LRH-stimulated (not the autonomous) secretion of LH and FSH. OB plus clomiphene had the same effect as OB alone. Clomiphene also raised the LRH-stimulated secretion of LH and FSH after pre-treatment with LRH, but OB did not do so: LRH prevented the stimulatory effect of OB but not of clomiphene. OB plus clomiphene had the same effect as OB alone. The absence of a stimulatory effect of OB on the LH-stimulated secretion of LH and FSH in the LRH-pre-treated rat appeared to be due to the very low gonadotropin content of the pituitary glands after pre-treatment with LRH and OB: the effect of OB on the LRH-responsiveness proper (i.e. release of LH and FSH as related to the pituitary LH and FSH content) remained stimulatory. Also clomiphene enhanced the LRH-responsiveness proper, but this drug cannot potentiate the gonadotropin stores-depleting effect of LRH. These results demonstrate that clomiphene exclusively ‘behave’s like an oestrogen-agonist, able to enhance the LRH-stimulated gonadotropin secretion. Also in the LRH-pre-treated rat clomiphene acts like an oestrogen-agonist, but unlike oestradiol clomiphene cannot potentiate the LH-induced depletion of the pituitary gonadotropin stores. Therefore, it can also raise the LRH-stimulated secretion of LH and FSH in the LRH-pre-treated OVX rat.

Clomiphene (-citrate), a triphenylethylene derivative, is frequently and successfully used in the treatment of anovulation (e.g. Jacobson et al. 1968; Ross et al. 1970).

However, the mechanism by which the drug induces ovulation is still not fully understood, although it is known that clomiphene has both oestrogenic and anti-oestrogenic properties. Like oestradiol, for instance, it can sensitize the gonadotrophic cells of the pituitary gland of the long-term ovariectomized (OVX) rat for the gonadotropin releasing activity of LRH (Hsueh et al. 1978; Adashi et al. 1981; Huang & Miller 1983; Schuiling et al. 1985a). However, the drug can
also compete with oestradiol at hypothalamic and pituitary receptor sites (Igarashi et al. 1968; Kato et al. 1968; Adashi et al. 1981). At this moment there is general consensus that apparently acting as an anti-oestrogen, clomiphene weakens the inhibitory effect of oestradiol on the secretion of LH and FSH. The ensuing rise of the LH and FSH secretion, then, would stimulate the ovaries and ovulation may then be induced (Vaitukaitis et al. 1971).

Recently we reported that oestradiol benzoate (OB), when given to OVX rats with chronically elevated plasma LH concentrations due to prolonged subcutaneous infusion of LRH, strongly depresses the LH and FSH responses induced by acute increments of LRH (negative effect of oestrogen), whilst in OVX rats not infused with LRH OB enhances such LH and FSH responses (positive effect of oestrogen). We therefore suggested that the nature of the effect of OB (negative or positive) may be fully determined by the LRH levels present (Schuiling et al. 1984a, 1984b).

In the present in vitro study we compared the effects of OB and of clomiphene on the autonomous (i.e. non-LRH-stimulated; Pasteels et al. 1977; Moes et al. 1983) and the LRH-stimulated components of LH and FSH secretion by pituitary glands of OVX rats which had for 5 days been pre-treated with LRH. In some of the experiments we investigated whether clomiphene can interfere with the negative effect of OB on the LH and FSH secretion.

Materials and Methods

Wistar rats were ovariectomized at the age of 3 months and used for experiments 2 weeks later. Ovariectomy was performed to eliminate the influence of ovarian hormones. The general arrangement of the experiments was as follows: some of the rats were infused with LRH for 5 days at the rate of 250 ng/h. LRH was delivered by Alzet® osmotic minipumps, model 2001, which were subcutaneously implanted at 09.00 h on day 0. Other rats received a 'sham-pump', i.e. a piece of silicone elastomer with the dimensions of a minipump.

On day 5 the rats were decapitated. The pituitary glands were quickly removed, and their in vitro rates of autonomous and supra-maximally LRH-stimulated release of LH and FSH were determined using a hemi-pituitary perfusion system according to a procedure described in Moes et al. (1983). After a 90-min pre-perfusion with medium only, the perfusion proper began: first medium containing no LRH was for 25 min pumped through the perfusion chambers in order to assess the autonomous secretion of LH and FSH; thereafter the pituitary glands were for 6 h exposed to medium containing LRH at the supra-maximally stimulating concentration of 1 µg/ml (cf Schuiling et al. 1984a). The concentration of LRH in the medium was chosen for methodological reasons: after the described LRH pre-treatment the pituitary glands of such rats will only respond significantly if they are subjected to a strong LRH-stimulus (cf Schuiling et al. 1984a, 1984b).

Both in the case of the LRH-pre-treatment and of the sham-procedure four different pre-treatments were given preceding the perfusion: I. clomiphene citrate, 100 µg by sc injection was given 72 and 24 h before perfusion; II oestradiol benzoate, 3 µg by sc injection, was given 72 and 24 h before perfusion; III. treatments I and III were combined; IV. solvent only (control rats).

LH and FSH were measured in the perfusion media and, after perfusion, in the pituitary glands by double antibody radioimmunoassay with NIADDK-rat LH/FSH-RP-1 as reference preparations.

The quantity of LH and FSH, present in the pituitary gland at the beginning of perfusion (tQ+C; µg LH/FSH per pituitary gland) was calculated by adding the total quantity of LH and FSH released during perfusion, tQ, and the LH/FSH content of the pituitary gland, still present at the end of perfusion, C (de Koning et al. 1981; Schuiling et al. 1985b). In a previous study we demonstrated that the parameter tQ+C is an adequate estimate of the quantities of LH and FSH present in the pituitary gland at the beginning of perfusion (Schuiling et al. 1984a).

Parameters; representation of the effects of OB and clomiphene; statistics

The effects of pre-treatment with OB, with clomiphene or with both OB and clomiphene on the autonomous and the supra-maximally LRH-stimulated secretion of LH and FSH by pituitary glands of control rats and by glands of rats also pre-treated with LRH were judged according to the following parameters: 1. the maximal rates of autonomous and supra-maximally LRH-stimulated LH/FSH secretion (ng LH/FSH per 5 min per pituitary gland); S_A and mS_A, respectively, and 2. the total quantity of LH and FSH secreted during perfusion, tQ.

The rates of autonomous LH/FSH secretion (S_A) were measured directly; the maximal supra-maximally LRH-stimulated LH/FSH secretion rates (mS_A) were calculated by subtraction of the autonomous LH/FSH secretion rates from the maximal 'total' secretion rates of LH and FSH (mS), which were also measured directly.

Thus: mS_A = mS_t - S_A.
The effects of OB, of clomiphene and of OB plus clomiphene on the parameters mentioned above were assessed by dividing the numerical value of a given parameter obtained with pituitaries of OB/clomiphene/ OB plus clomiphene-treated rats by the corresponding value of the matching oil-injected rats. We thus calculated ratios for tQ, S, and mSt.

In one series of experiments (see Results and Discussion) the total quantity of gonadotropin released during perifusion as well as the maximal rates of autonomous and supra-maximally LRH-stimulated LH/FSH secretion per 5 min were expressed as percentage of the pituitary LH/FSH content at the beginning of perifusion (cf Schuiling et al. 1985b). If one calculates ratios on the basis of these parameters of relative LH/FSH release, one gets an impression of the OB/clomiphene/ OB plus clomiphene-induced changes in the mode with which the pituitary gland expells LH and FSH from the gonadotrops, because these ratios are 'corrected' for changes in the pituitary LH/FSH content. The effect of OB, of clomiphene and of OB plus clomiphene was considered positive when the ratios were greater than 1.

Data are expressed as mean ± SEM. Statistical comparisons were made by analysis of variance and then by Duncan's multiple comparison test (Steele & Torrie 1960). A difference was considered to be significant when the analysis of variance showed significant heterogeneity for the whole group and the multiple comparison test gave a value of P < 0.05 for the two groups concerned.

**Results and Discussion**

Fig. 1 shows that a 3 days' treatment with either OB, with clomiphene or with OB plus clomiphene

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*,**, *, **: difference statistically significant (P < 0.05).
did with one exception not alter the LH and FSH contents of the pituitary gland; only the combined OB/clomiphene-treatment caused an increase of the pituitary FSH content. Fig. 1 also shows that after the present LRH pre-treatment (250 ng LRH/h for 5 days) the pituitary LH stores were significantly depleted and that after pre-treatment with LRH and OB the LH stores were even stronger depleted (cf Schuiling et al. 1984a, 1984b). Clomiphene did not potentiate the LRH-induced depletion of the pituitary LH stores. When LRH-pre-treated rats were injected with both OB and clomiphene, the pituitary LH stores were less severely depleted than after treatment with only OB. With LRH treatment the FSH stores showed in the oil-controls and after additional treatment with OB, with clomiphene and with both agents combined, comparable but less pronounced and in part statistically non-significant changes. These data agree with our previous observation that an LRH-depleted pituitary gland is not repleted as long as LRH-pre-treatment continues. Probably LRH can block the synthesis of LH and FSH (cf Schuiling et al. 1984a, 1984b, 1984c, 1985b).

Fig. 2 shows that the quantity of LH released by pituitary glands of OB-injected rats was significantly greater than that released by glands of oil-injected rats (OB/oil ratio: 2.0). There was no significant effect of OB on the secreted quantity of FSH (OB/oil ratio: 1.2). This dissimilar effect of OB on the secretion of LH and FSH can easily be explained when we look at the autonomous and the LRH-stimulated components of LH and FSH secretion separately. It then appears that OB had a significant positive effect on the autonomous secretion of LH (OB/oil ratio: 2.0), but not that of FSH (OB/oil ratio: 1.1). On the LRH-stimulated components of LH and FSH secretion, on the other hand, the effect of OB was strongly positive in both cases (OB/oil ratio for LH: 2.6 and for FSH: 2.2).

Clomiphene had no significant effect on the quantity of LH and FSH released during perfusion (clomiphene/oil ratio for LH: 1.4 and for FSH: 1.2). Like OB, clomiphene enhanced the LRH-stimulated (not: the autonomous) components of LH and FSH secretion (clomiphene/oil ratios 1.5 and 1.8, respectively): the drug acted as an oestrogen-agonist.

Also the effect of OB plus clomiphene on the in vitro release of LH and FSH was positive (see Fig. 2 for these and other OB plus clomiphene/oil data): clomiphene did not act as an anti-oestrogen.

After pre-treatment with LRH (250 ng/h for 5 days) the quantity of LH and FSH released during perfusion was with all modes of pre-treatment and all parameters significantly decreased. This was to be expected if one considers the significant depletion of the pituitary LH and FSH stores of these animals. In LRH-pre-treated rats OB did not enhance the quantity of LH and FSH released during perfusion; in the case of LH the effect of

| Parameters characterizing the secretion of LH (left panels) and FSH (right panels) during 6 h of perfusion of pituitary glands of OVX rats pre-treated with LRH (MP) or not pre-treated with LRH (Sh-P). Rats were also pre-treated with either solvent (□), with oestradiol benzoate (OB; ■, with clomiphene citrate (Cl; □) or with both OB and Cl (■), 72 and 24 h before perfusion. A) total quantity of LH and FSH secreted (µg LH/FSH per pituitary gland); B) autonomous 5 min secretion of LH and FSH (ng/LH/FSH 5 min per pituitary gland); C) maximal 1 µg/ml LRH-stimulated secretion of LH and FSH (ng LH/FSH per 5 min per pituitary gland).

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***; ***, ***: difference statistically significant (P < 0.05). Data on top of the bars: OB/oil; Cl/oil; OB + Cl/oil ratios.
OB was even inhibitory (negative) (OB/oil ratio: 0.7 for LH (cf the ratio of 2.0 in animals not pre-treated with LRH) and 1.1 for FSH). Probably this inhibitory effect is caused by the negative effect of the steroid on the pituitary LH content: if the quantity of LH released during perifusion was related to the pituitary LH content present at the beginning of perifusion (see Materials and Methods), and the OB/oil ratio was (re-)calculated accordingly, the ratio became 1.3. This indicates that also in the presence of LRH OB can still stimulate the secretory mechanisms of the gonadotrops.

That this stimulatory effect of oestradiol (which is in LRH-pre-treated rats smaller than in rats not pre-treated with LRH) is exerted on both the autonomous and the LRH-stimulated LH secretion can be seen when we relate the respective maximal 5-min rates of LH secretion to the pituitary content: whilst the pertinent OB/oil ratios calculated on the basis of the observed maximal 5-min secretion rates were 0.9 and 0.7, respectively, these ratios became 1.5 and 1.2 after 'correction' for the pituitary LH content. In the case of FSH the 'corrected' ratios became 1.0 (autonomous component) and 1.2 (LRH-stimulated component). In the foregoing mainly data concerning LH have been considered. The FSH data indicate that the control of FSH secretion does not differ from that of LH secretion. However, FSH secretion seems to be less 'oestradiol-sensitive'.

The effect of clomiphene on the quantity of LH and FSH secreted by pituitary glands of LRH-pre-treated rats differed markedly from that of OB: clomiphene did not only not depress the quantity of LH and FSH secreted during perifusion: it even enhanced it (clomiphene/oil ratio for LH: 2.2 and for FSH: 1.5). This is probably due to the fact that clomiphene, unlike OB, did not potentiate the LRH-induced depletion of the gonadotropin stores.

The effect of clomiphene plus OB on the LH/FSH release by pituitary glands of LRH-pre-treated rats generally took a somewhat intermediate position between the predominantly inhibitory effect of OB alone and the constantly stimulatory effect of clomiphene alone.

Our results demonstrate that in the present experimental situation – that is in the OVX rat – two effects of oestradiol determine the effect of oestradiol, namely: 1) potentiation of the gonadotropin stores-depleting effect of LRH (see Fig. 1), and 2) enhancement of the release of LH and FSH by the gonadotrops. With the present infusion rate of 250 ng LRH/h the former effect of oestradiol dominates so that it cannot raise the LH/FSH secretion and may even inhibit it (cf Schuling et al. 1985b).

Like oestradiol (but somewhat weaker) clomiphene increases gonadotropin release, but, unlike oestradiol, the drug does not potentiate the LRH-induced depletion of the gonadotropin stores. Hence also in the LRH-pre-treated OVX rat clomiphene enhances the secretion of LH and FSH. Our results thus demonstrate that neither in rats not pre-treated with LRH, nor in rats pre-treated with LRH clomiphene 'behaves' like an oestrogen-antagonist: in our experimental models we could only demonstrate oestrogen-agonistic properties of clomiphene.

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