Effect of in vivo pre-treatment with oestradiol and either GnRH, GnRH agonistic analog or GnRH antagonistic analog on GnRH-stimulated secretion of LH in vitro

G. A. Schuiling, T. R. Koiter and H. Moes

Division of Reproductive Biology, Department of Obstetrics and Gynaecology, University of Groningen, Oostersingel 59, NL-9713 EZ Groningen (The Netherlands)
Received 19 October 1990; accepted 4 January 1991

Summary. In vivo treatment with GnRH or with GnRH agonistic analog (AG), but not with GnRH antagonistic analog (ANT), depleted the LH stores of the rat pituitary gland. This depletion was potentiated by oestradiol. Oestradiol augmented the in vitro LH response of the pituitary gland to GnRH. This augmenting effect of oestradiol became smaller with increasing rates of in vivo administration of GnRH or AG, but not with ANT. With respect to both depletion of the LH stores and suppression of the augmenting effect of oestradiol, AG ist about 20 times as potent as GnRH.
Key words. GnRH; GnRH agonist; GnRH antagonist; oestradiol; pituitary gland; LH response.

Oestradiol influences the gonadotropin response of the pituitary gland to gonadotropin-releasing hormone (GnRH). This influence of oestradiol can be positive or negative and may change over time. In ovariectomized (OVX) rats the response is depressed immediately after administration of oestradiol[1] but becomes augmented after some time[2]. The response remains depressed in rats with high serum GnRH levels, but increases as soon as the GnRH levels decrease[3]. These data demonstrate that the effect of oestradiol on the gonadotrophs of the pituitary gland is controlled by GnRH.

In this in vivo/in vitro study we investigated whether GnRH analogs, too, influence the effect of oestradiol on the pituitary gland. OVX rats were treated for 6 days with either GnRH, 'superpotent' agonistic GnRH analog (AG), or antagonistic GnRH analog (ANT), and with oestradiol. LH responses were induced by GnRH in vitro with hemipituitary glands.

Materials and methods

Animals. Two week ovariectomized Wistar rats (age 14–16 weeks; about 200 g) were used. Ovariectomy was performed in order to prevent ovarian hormones affecting the response of the pituitary gland to exogenous hormones. Operations (insertion of osmotic minipumps and of Silastic implants, Dow Corning, Midland, MI, USA) were performed under ether anaesthesia. Rats were killed by decapitation.

In vivo treatments. In some of the rats, osmotic minipumps (Alzet model 2001; Alza Corp., Palo Alto, CA, USA) were implanted s.c.; other rats received a Silastic 'sham pump', i.e., a piece of silicone elastomer with the dimensions of a minipump. The day of implantation was day 0 of the experiments. Minipumps released either GnRH for 6 days at 25, 50, 100, 250 or 500 ng/h, or agonistic analog of GnRH (D-Ser(Bu^t)^6-des-Gly^{10}-GnRH-ethylamide; Buserelin®[4, 5]), at 2.5, 5, 10.0, 25 and 100 ng/h and in a volume of 1 µl/h. This dose-range was chosen because as an LH releasing factor, Buserelin is 10–100 times as potent as GnRH[6]. Another group of rats received antagonistic analog of GnRH (Ac-D-4Cl-Phe^1-D-4-Cl-Phe^2-D-Trp^3-D-Phe^6-D-Ala^{10}-GnRH; Org 30093[7, 8], a gift of Organon Ltd, Oss, The Netherlands). ANT was dissolved in saline/polyethylene-glycol-400 and injected s.c. at 09.00 h on days 0–5 (100 µg/injection of 0.2 ml). This dose of ANT is 10 times as high as the minimal dose required for blockade of ovulation in the rat[9]. Control rats were injected with solvent only. Oestradiol benzoate, dissolved in arachis oil, was injected s.c. at 09.00 h on days 3 and 5 (3 µg/injection of 0.2 ml).

Perifusion of the pituitary gland. After decapitation, at 09.00 h on day 6, the pituitary glands were quickly removed for perifusion as previously described[9]. Briefly, after removal of the pituitary glands the glands were cut in half and washed for 10 min at 37 °C in a perifusion medium (Krebs-Ringer bicarbonate buffer containing glucose and 1% (w/v) bovine serum albumin) gassed with 95% O_2/5% CO_2. Thereafter, they were transferred to perifusion chambers, with two hemipituitary glands (from different animals) per chamber. The volume of the chambers was about 300 µl. Gassed medium at 37 °C was pumped through the chambers at 134 ± 1.4 µl/min using a peristaltic pump. The perifusion was started after a 90-min pre-perifusion with medium only.

First, medium without GnRH was pumped through the chambers for 25 min to assess the basal secretion of LH; thereafter the pituitary glands were exposed to medium containing GnRH at the concentration of 1 µg/ml. Samples were collected over 5-min intervals. The high stimulating concentration of 1 µg GnRH/ml in the perifusion medium was chosen because owing to the GnRH (-analog) treatment in vivo, only a high concentration of GnRH is capable of inducing measurable LH responses in vitro.

Measurement of LH. The two hemipituitary glands from each perifusion chamber were homogenized in saline at 0 °C using a Brown Potter homogenizer (final volume: about 2.5 ml). The homogenate was centrifuged at 2000 × g for 15 min at 4 °C. The pellet was re-suspended in 1 ml of saline and again centrifuged at 2000 × g

(10 min; 4 °C). The supernatants were combined and the volume was adjusted with saline to 5 ml. The extract was stored at − 20 °C until assay of LH. The LH contents of the pituitary glands after perifusion and the LH concentrations in the perifusion media were measured by double antibody radioimmunoassay with anti-ovine LH as antiserum and rat LH as tracer[10]. LH-RP-1, donated by the NIADDK, was the reference preparation.

Parameters; statistics. The in vitro GnRH-stimulated release of LH was expressed in ng per 5 min per pituitary gland. The maximal rate of GnRH-induced LH secretion was calculated by subtraction of the rate of non-GnRH-stimulated LH secretion from the 'total' rate of LH secretion. The quantity of LH present in the pituitary gland at the beginning of perifusion was estimated by adding the pituitary LH content still present at the end of perifusion and the total quantity of LH released during perifusion[11].

Data are expressed as means ±SEM. Statistical comparisons were made by analysis of variance (Kruskal-Wallis; subsequently groups were compared in twos by the Mann-Whitney U-test[12]). Differences were considered to be statistically significant when $p < 0.05$.

Results and discussion

Figure 1 shows that a 6-day treatment with GnRH or AG caused a dose-dependent depletion of the pituitary LH stores. Oestradiol potentiated the GnRH/AG-induced depletion in animals which had been pretreated in vivo with 100 µg GnRH or more or with 10 µg AG or more. ANT had no effect on the LH content of the pituitary gland, either alone, or in combination with oestradiol.

It was found by interpolation that the in vivo infusion rates of GnRH and AG necessary to reduce the LH stores by 50% (ED-50) amounted to about 100 ng GnRH/h and 5 ng AG/h in rats not treated with oestradiol and about 75 ng GnRH/h and 5 ng AG/h in rats pretreated with oestradiol. On the basis of these ED-50s an AG/GnRH potency ratio was calculated. This ratio was 15–20.

Figure 2 shows that after 6 days of pretreatment with GnRH or AG there was an inverse relationship between the in vivo rate of GnRH/AG infusion and the maximal rates of GnRH-induced in vitro LH secretion. Moreover, there was a positive effect of oestradiol on the LH response. This effect decreased with increasing in vivo GnRH/AG infusion rates and became negative at the higher GnRH/AG infusion rates. Interpolation revealed that at about 160 ng GnRH/h and 7.0 ng AG/h the effect of oestradiol on the LH response was zero. The AG/GnRH potency ratio calculated on the basis of these data was about 23.

Figure 2 also shows that in vivo treatment with ANT suppressed the in vitro LH secretion by about 45%. This indicates that during perifusion the antagonist still (partly) occupied the GnRH receptors of the gonadotrophs.

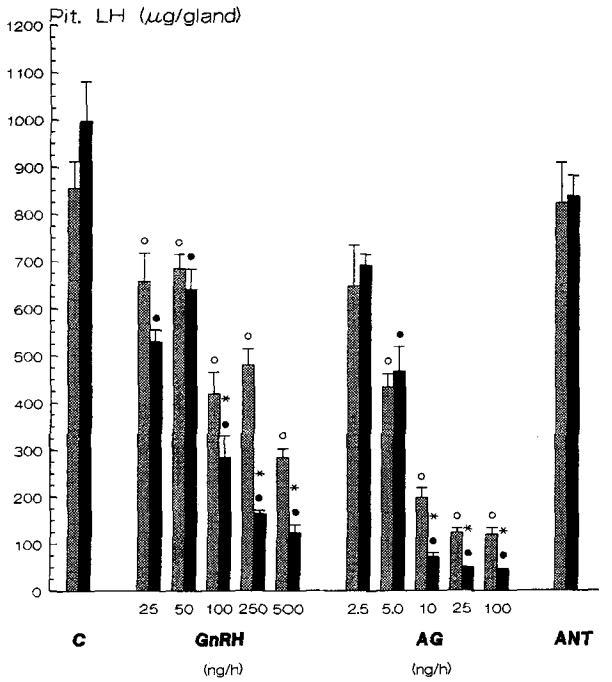

Figure 1. Pituitary content of LH in ovariectomized rats on day 6 of treatment with GnRH, GnRH agonistic analog, AG, or GnRH antagonistic analog, ANT. GnRH and AG were s.c. infused at 0, 25, 50, 100, 250 and 500 ng/h and 2.5, 5, 10, 25 and 100 ng/h, respectively; ANT was injected s.c. at 100 µg/day. N = 3–7. Rats were injected with either oil (hatched bars) or oestradiol benzoate (closed bars). Values are means ± SEM.

For the effect of GnRH and AG: ○, ● $p < 0.05$ compared with control value (ANOVA and Mann-Whitney U-test); for the effect of oestradiol benzoate: *$p < 0.05$ compared with oil-treated rats at the same dose of GnRH or AG (one-sided) or ANT (two-sided) (Mann-Whitney U-test).

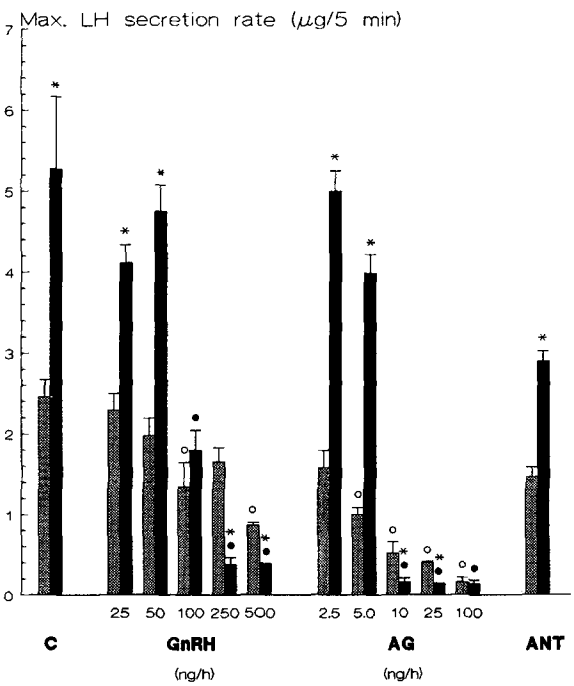

Figure 2. Maximal GnRH-induced secretion of LH by perifused anterior pituitary tissue from ovariectomized rats. The concentrations of GnRH in the medium was 1 µg/ml. Rats had been pretreated for 6 days with GnRH, GnRH agonistic analog, AG, or GnRH antagonistic analog, ANT. See further: legend to figure 1.

Still, ANT did not affect the effect of oestradiol. We therefore conclude that in contrast to GnRH and AG, ANT did not prevent the augmenting effect of oestradiol. Although both GnRH, AG and ANT firmly bind to the GnRH receptors of the gonadotrophs [13], receptor binding is not sufficient to exhibit intrinsic GnRH activity: for this the receptor-ligand complex must be internalized [14, 15]. GnRH antagonists are not internalized after binding to the GnRH receptor [16]. The present results therefore suggest that, like induction of LH release, prevention of the augmenting effect of oestradiol requires internalization of the GnRH/Ag-receptor complex. Interaction between GnRH/AG and oestradiol, therefore, probably does not take place at the level of the binding of ligands to the GnRH receptor.

Probably it is not a question of binding of ligands to the oestrogen receptor, either, as we demonstrated recently that the positive effect of the non-steroidal oestrogen analog clomiphene on the GnRH-induced LH response is *not* prevented by GnRH [17], in spite of the fact that this drug firmly binds to the oestrogen receptor [18].

We conclude that superpotent GnRH agonists are also superpotent with regard to prevention of the positive effect of oestradiol, and the GnRH antagonists are ineffective in this respect. We suggest that GnRH/AG-oestradiol interaction does not take place at the level of the receptors of GnRH or oestradiol.

1 Schuiling, G. A., and Gnodde, H. P., J. Endocr. *72* (1977) 121.
2 Schuiling, G. A., and Gnodde, H. P., J. Endocr. *71* (1976) 1.
3 Schuiling, G. A., Moes, H., and Koiter, T. R., Acta endocr. *110* (1985).
4 Sandow, J., von Rechenberg, W., Kuhl, H., Baumann, R., Kraus, B., Jerzabek, G., and Kille, S., Hormone Res. *11* (1979) 303.
5 Sandow, J., von Rechenberg, W., Jerzabek, G., Engelbart, K., Kuhl, H., and Fraser, H., Acta endocr. *94* (1980) 489.
6 Koiter, T. R., Moes, H., and Schuiling, G. A., Acta endocr. *119* (1988) 345.
7 Erchegyi, J., Coy, D. H., Nekola, M. V., Coy, E. J., Schally, A. V., Mezo, I., and Teplan, I., Biochem. biophys. Res. Commun. *100* (1981) 915.
8 Meys-Roelofs, H. M. A., Kramer, P., van Cappellen, W. A., and Schuiling, G. A., J. Endocr. *112* (1987) 407.
9 Moes, H., Koiter, T. R., and Schuiling, G. A., IRCS med. Sci. *11* (1983) 310.
10 Welschen, R., Osman, P., Dullaart, J., de Greef, W. J., Uilenbroek, J. Th. J., and de Jong, F. H., J. Endocr. *64* (1975) 37.
11 Schuiling, G. A., Moes, H., and Koiter, T. R., Acta endocr. *106* (1984) 454.
12 Sokal, R. R., and Rohlf, F. J., Biometrics: the Principles and Practice of Statistics in Biological Research, 2nd edn. Freeman, W. H. and Co., San Francisco 1981.
13 Koiter, T. R., Denef, C., Andries, M., Moes, H., and Schuiling, G. A., Life Sci. *39* (1986) 443.
14 Catt, K. J., Loumaye, E., Katikinene, M., Hyde, C. L., Childs, G., Amsterdam, A., and Naor, Z., in: Role of Peptides and Proteins in Control of Reproduction, p. 33. Eds S. M. McCann and D. Dhindsa. Elsevier, Amsterdam 1983.
15 Loumaye, E., and Catt, K. J., Science *215* (1982) 983.
16 Loumaye, E., Wynn, P. C., Coy, D. H., and Catt, K. J., J. biol. Chem. *259* (1984) 12663.
17 Schuiling, G. A., Pols-Valkhof, N., and Koiter, T. R., Experientia *41* (1985) 1060.
18 Jordan, V. C., Pharmac. Rev. *36* (1984) 245.

0014-4754/91/070716-03$1.50 + 0.20/0

EGF receptor induction and insulin-EGF overlap in *Tetrahymena*

G. Csaba and P. Kovács

Department of Biology, Semmelweis University of Medicine, H-1445 Budapest (Hungary)
Received 22 October 1990; accepted 28 January 1991

Summary. Offspring generations of *Tetrahymena* pretreated (imprinted) with insulin showed a greater binding capacity for the hormone than offspring of untreated ones. The epidermal growth factor (EGF) imprinted for insulin to a greater degree than insulin itself, and vice versa: insulin imprinted for EGF more efficiently than EGF itself. These phenomena can be explained by the overlap of insulin and EGF on one another's receptors in *Tetrahymena*.
Key words. Tetrahymena; EGF; insulin; hormonal imprinting; receptors.

Although the hormone receptors of higher organisms are genetically encoded, they begin to function only after adaptation to the appropriate hormone [1]. Receptor adaptation (amplification) takes place as a rule in the early postnatal period, when primary interaction with the appropriate hormone gives rise to hormonal imprinting that accounts for stabilization of the binding capacity characteristic of adulthood [2]. Unicellular organisms do not have encoded receptors, but possess certain membrane structures which are able to recognize environmental signal molecules. These structures can be imprinted by hormones of higher vertebrates, responding to these by modification of the binding capacity and of certain functional parameters [3, 4]. It follows that hormonal imprinting can also take place at the unicellular level and account for formation of genuine receptors which persist over many offspring generations. For example, primary interaction of the unicellular organism *Tetrahymena* with insulin resulted in the formation of specific insulin receptors, as substantiated by displacement and saturation experiments [5]. The insulin molecule displayed a full imprinting potential also after deprivation of its 5-C termi-