DESENSITIZATION OF GONADOTROPIN-RELEASING RESPONSE FOLLOWING VAGINAL CONSECUTIVE ADMINISTRATION OF LEUPROLIDE IN RATS

HIROAKI OKADA, IWAO YAMAZAKI, YASUFUMI SAKURA, TAKATSUKA YASHIKI, TSUGIO SHIMAMOTO AND HIROYUKI MIMA

Central Research Division, Takeda Chemical Industries, Ltd., 2-17-85 Juso, Yodogawa, Osaka, 532, Japan

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The vaginal absorption of a potent luteinizing hormone-releasing hormone analog (leuprolide), and the gonadotropins (luteinizing hormone and follicle-stimulating hormone)-releasing response after continuous vaginal and subcutaneous administration of the analog to rats were determined by the radioimmunoassay. A marked suppressive effect on the gonadotropin-releasing response along with long-lasting serum levels of leuprolide were paradoxically observed after consecutive 3-d vaginal administration of the analog at doses of 1 $\mu\text{g}/\text{kg}$ or greater. Pituitary function recovered progressively with cessation of the treatment but was not complete 4 d after cessation. Continuous vaginal and subcutaneous infusion resulted in an almost complete inhibition of both gonadotropins response. It is suggested that effective desensitization of the pituitary gonadotropin response is elicited by continuous administration of leuprolide and, therefore, the vaginal administration resulting in prolonged serum levels of the analog could be preferable as a self-administration method for medical treatments such as anti-tumor therapy and birth control.

Keywords — leuprolide; LHRH analog; vaginal administration in rat; gonadotropin response; desensitization

INTRODUCTION

In a previous study,¹⁾ we demonstrated by radioimmunoassay that vaginal administration of a potent luteinizing hormone-releasing hormone (LHRH) analog, des-Gly¹⁰-(D-Leu⁶)-LHRH ethylamide: leuprolide, with citric acid resulted in high and long-lasting serum levels of the analog and gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH); the analog disappeared rapidly from the serum after intravenous injection. A linear dose-absorption correlation was observed at a dose range of 0.1 to 1000 $\mu\text{g}/\text{kg}$, whereas, gonadotropin-releasing response attained a plateau above 10 $\mu\text{g}/\text{kg}$. Moreover, the estrous cycle stages markedly altered vaginal absorption but the cyclic variation was eliminated by continuous pretreatment with the analog. Thus, it was proposed that vaginal administration was a potentially effective method for anti-cancer therapy with leuprolide.

Recently, it was reported that a single high dose or chronic administration of potent LHRH analogs or gonadotropins caused paradoxically a marked inhibitory effect on gonadotropin-releasing response in the pituitary, functional gonadal atrophy,²⁻¹²⁾ reduction of receptors, “down regulation,” of the gonadotropins in the ovary¹³⁻¹⁷⁾ and testis,^{13,18-24)} and of LHRH in the ovary²⁵⁾ and pituitary.²⁶⁾ Desensitization or down regulation by chronic treatment with potent LHRH analogs suggest potential new applications for these compounds such as birth control agents in both sexes,²⁷⁻³⁰⁾ treatment of idiopathic precocious puberty,^{31,32)} and anti-cancer therapy against hormone-dependent tumors,^{33,34)} in addition to conventional therapies: ovulation-induction,³⁵⁾ and treatment of amenorrhea,³⁶⁾ oligospermia,³⁷⁾ and cryptorchidism.³⁸⁾

In the present study, to obtain information for a rational dosage regimen of leuprolide in anti-

cancer therapy, gonadotropin-releasing responses and serum levels of leuprolide were determined after consecutive daily administration or continuous infusion of the analog by vaginal and subcutaneous routes. The recovery from pituitary refractoriness and the dose-response correlation after the consecutive daily administration were also investigated.

MATERIALS AND METHODS

Animals and Materials — Mature female Sprague-Dawley rats (Clea Japan, Co., Tokyo) aged 120 to 150 d, weighing 250 to 330 g, and exhibiting two or more consecutive 4-d estrous cycles on daily examination of vaginal smears were used at diestrus. For multiple doses, administration was commenced on diestrus.

Leuprolide used were of the same quality reported in the previous study.¹⁾

Radioimmunoassay of Leuprolide and Gonadotropins — Leuprolide and gonadotropins in rat sera were determined in duplicate by double antibody radioimmunoassay.¹⁾ For the assay of gonadotropins, kits supplied by the National Institute of Arthritis, Metabolic and Digestive Diseases (NIAMDD, Bethesda, MD, U.S.A.) were used, and values are expressed in terms of the standard rat gonadotropins (NIAMDD-Rat-LH RP-1 and NIAMDD-Rat-FSH RP-1) respectively. Serum samples were stored at -20°C until the day of assay, and dilution was made with 0.01M phosphate buffer (pH 7.2) before the assay.

Statistical evaluation of the data was performed using Student's *t*-test.

Consecutive Vaginal and Subcutaneous Administration — Leuprolide was administered subcutaneously, 100 $\mu\text{g}/\text{kg}$ and vaginally, 150 $\mu\text{g}/\text{rat}/50\text{ mg}$ in an oleaginous suppository (Witepsol S55, Dynamit Nobel Aktiengesellschaft, W. Germany) containing 10% citric acid, once a day for 3, 7, or 14 d. After the last dose, blood was collected from the tail vein to determine gonadotropin serum levels.

To determine serum leuprolide levels, the analog was administered vaginally at a dose of 500 $\mu\text{g}/\text{kg}/400\text{ mg}$ of 5% methylcellulose jelly

containing pH 3.5, 5% citric acid solution (solution A) once a day for 3, 7, or 14 d. Histological examination was carried out on ovaries and vagina after collection of the blood. Changes in the estrous cycle following the consecutive subcutaneous injection were determined by cytological examination of vaginal smears.

Recovery of Pituitary Gonadotropin Response

— Leuprolide, at a dose of 100 $\mu\text{g}/\text{kg}/\text{d}$, was administered subcutaneously to rats for 3 d and, after 1, 2 or 3 d of rest, a second subcutaneous administration at the same dose was given.

Dose-response Following Consecutive Administration — Leuprolide was administered subcutaneously, at doses of 0.1, 1, 10, and 100 $\mu\text{g}/\text{kg}$, and vaginally, at doses of 0.1, 1, 10, 100, and 500 $\mu\text{g}/\text{kg}/400\text{ mg}$ of the jelly containing solution A, once a day for 14 d. Blood was collected 2 h after administration every other day, and 0, 1, 4, and 6 h after the last (14th day) dose under pentobarbital (50 mg/kg, intraperitoneally) anesthesia.

Continuous Infusion by Vaginal and Subcutaneous Routes — Leuprolide was infused continuously into diestrous (initial stage) rats weighing $300 \pm 5\text{ g}$ with an osmotic minipump (Alzet, Model 1701, pumping rate = $1\mu\text{l}/\text{h}$, Alza Co., Palo Alto, CA, U.S.A.) filled with the analog, 30 $\mu\text{g}/24\mu\text{l}$ in saline for the subcutaneous route, and 150 $\mu\text{g}/24\mu\text{l}$ in solution A for the vaginal route. The minipump was implanted subcutaneously in the dorsal cervical site, or inserted into the vagina by setting the outlet of the pump toward the uterocervical canal under the pentobarbital anesthesia; the orifice of the vagina was sutured. Blood was collected from the tail vein 0, 2, 6, 24, 48, 72, 96, 120, 144, and 150 h after administration. The vagina and ovaries were removed for histological examination after the experiment was terminated.

RESULTS

Consecutive Vaginal and Subcutaneous Administration

Serum levels and AUC (area under the serum level-time curve) increment (ΔAUC) of

gonadotropin at 6 h after the last dose of consecutive subcutaneous and vaginal administration of leuprolide for 3, 7, or 14 d are shown in Figs. 1 and 2, respectively. Administration by either route significantly suppressed the releasing response of both gonadotropins by the 3rd day of

treatment (significantly different from single dose, $p < 0.01$). The *AUC* increment following the 3-d treatment was 11.6% for luteinizing hormone (LH) and 27.4% for follicle-stimulating hormone (FSH) by the subcutaneous route, and 5.6% for LH and 19.1% for FSH by the vaginal

FIG. 1. Serum Gonadotropin Levels after the Last Dose of Subcutaneous and Vaginal Administration of Leuprolide for 3, 7, or 14 d in Rats.

The analog was administered subcutaneously, 100 $\mu\text{g/kg}$, (○), and vaginally, 150 $\mu\text{g/rat}$ in an oleaginous suppository containing 10% citric acid, (●) to rats exhibited diestrus initially. Each point represents the mean $\pm$ SE of three rats.

route (ΔAUC after a single dose is 100%). A more marked suppression in the LH releasing response than in the FSH response was observed, and vaginal administration produced a stronger suppression.

Smear examination revealed that the rats were in diestrus directly or after one normal cycle during the 7 or 14-d treatment period. During the treatment, most of the smears were muciferous.

FIG. 2. AUC Increment (ΔAUC) of Gonadotropins in 6 h after the Last Dose of Consecutive Subcutaneous and Vaginal Administration of Leuprolide for 3, 7, or 14 d in Rats

The experiment was performed as described in Fig. 1. Each bar represents the mean $\pm$ SE of three rats. a) significantly different from single dose by Student's *t*-test ($p < 0.05$), b) ($p < 0.01$).

▨ subcutaneous, □ vaginal.

Normal estrous cycling was resumed 3 d after cessation of the treatment in all rats following 7 and 14 d administration.

Serum levels of leuprolide after consecutive daily vaginal administration for 3, 7, or 14 d are shown in Fig. 3. AUC s of the analog after 3 or 7-d treatment were almost equivalent to that after the initial dose but that after 14-d treatment was reduced by 35%. Histological observation in 14-d treated rats showed a relatively thick epithelium (7–9 layers) with stratum corneum and infiltration of neutrophils in the vagina, and a numeral increase in developed corpora lutea without the presence of Graafian follicle in the ovaries.

Recovery of Pituitary Gonadotropin Response

The AUC increment of gonadotropins in 6 h following subcutaneous administration of leuprolide, 100 $\mu\text{g}/\text{kg}$, after 1, 2, or 3 d of rest subsequent to a 3-d pretreatment are shown in Fig. 4. The reduced responses (LH: 11.6%, FSH: 27.4%) following the initial 3-d treatment recovered progressively with increasing periods for rest; LH: 41.3%, FSH: 41.0% after 1-d interval, and LH: 65.2%, FSH: 77.4% after a 3-d interval.

Dose-response Following Consecutive Administration

Serum gonadotropin levels following consecutive subcutaneous (0.1–100 $\mu\text{g}/\text{kg}$) and vaginal (0.1–500 $\mu\text{g}/\text{kg}$) administration of leuprolide are shown in Figs. 5 and 6. The serum levels 2 h after dosing corresponded to the peak time in both subcutaneous and vaginal administration. The releasing response of LH attained the peak above 1 $\mu\text{g}/\text{kg}$ and 10 $\mu\text{g}/\text{kg}$ of leuprolide after single subcutaneous or vaginal administration, respectively, whereas, a markedly reduced response was observed above 0.1 $\mu\text{g}/\text{kg}$ and 1 $\mu\text{g}/\text{kg}$ after consecutive subcutaneous or vaginal administration. Both routes had less inhibitory effect on the FSH response; the response during the subcutaneous administration was about two thirds of that at first dose and the response during vaginal administration was a half. A stronger inhibitory effect on both gonadotropins response during the consecutive administration was exerted by the

FIG. 3. Serum Leuprolide Levels after the Last Dose of Consecutive Vaginal Administration for 3, 7, or 14 d in Rats

The analog was administered at a dose of 500 $\mu\text{g}/\text{kg}$ in 5% methylcellulose jelly containing solution A. Each point represents the mean $\pm$ SE of six rats. a) significantly different from single dose ($p < 0.05$).

FIG. 4. AUC Increment (ΔAUC) of Gonadotropins in 6 h Following a Subcutaneous Administration of Leuprolide after 1, 2, or 3 d of Rest to Rats with a 3-d Treatment

The analog was administered at a dose of 100 $\mu\text{g}/\text{kg}/\text{d}$. Each bar represents the mean $\pm$ SE of three rats. a) significantly different from single dose ($p < 0.05$), b) ($p < 0.01$).

vaginal routes. The dose-gonadotropin responses after single and 14 consecutive days vaginal administration of the analog are plotted in Fig. 7. An obvious inhibitory effect on the LH and FSH responses by consecutive administration was elicited at doses of 1 $\mu\text{g/kg}$ or greater of the analog, except the FSH response at the dose of 100 $\mu\text{g/kg}$ which rebounded after 7th day (Fig. 6).

Continuous Vaginal and Subcutaneous Infusion

Serum levels of gonadotropins and leuprolide following the subcutaneous, 30 $\mu\text{g/rat/d}$, and vaginal, 150 $\mu\text{g/rat/d}$, continuous infusion by the

osmotic minipump are shown in Figs. 8 and 9. The gonadotropins responses by both routes were almost identical; a potent response was elicited during the first day, but, thereafter, a marked suppression was observed in both gonadotropins, and levels corresponded to those before treatment.

Serum leuprolide levels following continuous subcutaneous infusion rapidly attained a steady state after 6 h and a constant serum level was maintained at 6.56 (5.04–8.71) ng/ml for 6 d. Vaginal infusion also provided rapid absorption, and high and sustained serum levels of the analog were observed at 8.18 (3.09–14.4) ng/ml for 6 d.

FIG. 5. Serum Gonadotropin Levels Following Consecutive Daily Subcutaneous Administration of Leuprolide at Different Doses in Rats

The analog was administered at a dose of 0.1 $\mu\text{g/kg}$ ($\blacktriangle$), 1 $\mu\text{g/kg}$ ($\triangle$), 10 $\mu\text{g/kg}$ ($\circ$), and 100 $\mu\text{g/kg}$ ($\square$) to rats exhibited diestrus initially. Each serum level shows the value at 2 h after the dose and represents the mean $\pm$ SE of five rats.

FIG. 6. Serum Gonadotropin Levels Following Consecutive Daily Vaginal Administration of Leuprolide at Different Doses in 5% Methylcellulose Jelly Containing Solution A in Rats

The analog was administered at a dose of 0.1 $\mu\text{g/kg}$ ($\blacktriangle$), 1 $\mu\text{g/kg}$ ($\triangle$), 10 $\mu\text{g/kg}$ ($\circ$), 100 $\mu\text{g/kg}$ ($\square$) and 500 $\mu\text{g/kg}$ ($\bullet$) to rats exhibited diestrus initially. Each serum level shows the value at 2 h after the dose and represents the mean $\pm$ SE of five rats.

Histological observation by both routes revealed infiltration of neutrophils, thin epithelium without any cornification in the vagina, numeral increase in large corpora lutea devoid of follicular development, and reduction in size of the ovaries.

DISCUSSION

Consecutive daily administration of leuprolide

by subcutaneous and vaginal routes caused a marked inhibitory effect of gonadotropins secretion of the pituitary, which persisted to the same degree during treatment for 14 d, and was evident 3 d after commencement of the treatment. Equivalent absorbability of leuprolide among the single, 3, or 7-d consecutive vaginal administration was recognized although, after the 14-d treatment, a slight but significant decrease in absorption was observed. Histological observation indicated a prolonged stimulation on the anterior pituitary: halt of the estrous cycle at diestrus and functional atrophy of the ovaries. These results

FIG. 7. Dose-response Correlation Curves of Leuprolide after Single and 14 Consecutive Days Vaginal Administration to Rats

The response is shown by AUC increment (ΔAUC) of gonadotropins in 6 h after single (○) and 14 consecutive days (●) administration. Experiment of consecutive administration was performed as described in Fig. 6. Each point represents the mean $\pm$ SE of five rats.

FIG. 8. Serum Gonadotropin Levels following Continuous Subcutaneous and Vaginal Infusion of Leuprolide during 6 d in Rats

The analog was administered subcutaneously, 30 μg/rat/d, (○) and vaginally, 150 μg/rat/d in solution A, (●) by an osmotic minipump. Each point represents the mean $\pm$ SE of five rats.

reveal that the marked reduction of gonadotropins release after vaginal administration of leuprolide is attributable not to the decrease of absorption but to the responsiveness of the target organ. Down regulation of LHRH receptors in the pituitary²⁶⁾ rather than depletion of pituitary gonadotropins,^{3,5,7)} as observed in chronic treatment of insulin,³⁹⁾ thyrotropin-releasing hormone,^{40,41)} and human growth hormone,⁴²⁾ is a more plausible mechanism for decreased gonadotropin response. The reason for the slight decrease of vaginal absorption after 14 consecutive d of administration is ambiguous at present, but might cause from the 2–3 fold thicker vaginal epithelium with the presence of a stratum corneum than that seen in the subcutaneously treated rats, which was most likely induced by the persistent stimulation of the glass inserter or cotton ball.

As the maximum desensitization in rat pituitary should be attained in 3 d, based on the reduction of LHRH and LH receptors in the rat ovary²⁵⁾ and LH receptors of the rat testis,²⁰⁾ recovery of the gonadotropin releasing responsiveness of the pituitary was determined following the 3-d consecutive treatment. The results reveal that the gonadotropin responsiveness

progressively recovered but was not complete after a 3-d rest. Such slow reversal of the down regulatory effects of the LHRH analogs was also reported in the ewe (about 92 h),²⁾ in LH receptors of the rat testis (8 d),²⁰⁾ and in LH receptors of the rat ovary (more than 5 d).²⁵⁾ On the other hand, cytological examination of vaginal smears showed that estrous cycling, halted at diestrus by consecutive treatment with leuprolide for more than 5 d, was resumed 3 d after cessation of the treatment. From these findings, it is suggested that desensitization in the rat pituitary can be maintained for a one or 2-d interval, but consecutive daily treatment results in a stronger desensitization.

The marked inhibitory effect on LH releasing response was exerted after consecutive administration of leuprolide by subcutaneous (above 0.1 $\mu\text{g/kg}$) and vaginal (1 $\mu\text{g/kg}$) routes, whereas, that on FSH response was not so marked, but was obvious at doses of 1 $\mu\text{g/kg}$ or greater by both routes. It appeared that the stronger inhibitory effect of these responses during consecutive administration was elicited by the vaginal route. This may ascribed to the long-lasting serum level of the analog.¹⁾ These results are in good agreement with the doses inducing down regulation reported previously: rat pituitary LHRH receptors were reduced by daily treatment of 1 $\mu\text{g/rat}$ (*ca.* 5 $\mu\text{g/kg}$) of D-Trp⁶-LHRH,²⁶⁾ and reduction of testicular LH receptors was recognized by the repeated subcutaneous administration of leuprolide at doses of 0.2 $\mu\text{g/rat}$ (*ca.* 1 $\mu\text{g/kg}$)²¹⁾ and 0.6 $\mu\text{g/rat}$.²⁰⁾ Considering that the minimum dose of leuprolide eliciting the anti-tumor activity against 7,21-dimethylbenz[*a*]anthracene-induced rat mammary tumors was about 100 $\mu\text{g/kg}$ by the vaginal route,⁴³⁾ a much larger dose seems to be necessary for the anti-tumor activity accompanied by ovarian atrophy, "chemical ovariectomy",³⁴⁾ than for desensitization of gonadotropin response in the pituitary.

Continuous vaginal and subcutaneous infusion of leuprolide by an osmotic minipump maintained the high and sustained serum levels of the analog, and showed a drastic inhibitory effect on

FIG. 9. Serum Leuprolide Levels Following Continuous Subcutaneous and Vaginal Infusion during 6 d in Rats

The analog was administered subcutaneously (○) and vaginally (●) as described in Fig. 8. Each point represents the mean $\pm$ SE of five rats.

releasing responses of both gonadotropins. This indicates that continuous stimulation of the target organs with the analog exerts a much stronger desensitization than the pulsatile stimulation. Similar result can be found in the inhibitory effect of gonadotropin-releasing response in the rhesus monkey,⁸⁾ and in the down regulation of rat ovarian LHRH receptors.²⁴⁾ The progressive elevation of the serum level of leuprolide (2 ng/ml/d) after vaginal infusion may be the same phenomena as the enhancement of vaginal absorption following consecutive subcutaneous pretreatment with the analog,¹⁾ since a similar thin vaginal epithelium was observed after vaginal infusion as was seen in the subcutaneous pretreatment.

In summary, consecutive daily vaginal administration of leuprolide exerted a marked inhibitory effect of gonadotropins-releasing response at doses of 1 µg/kg or greater possibly due to the prolonged serum levels of the analog. Although pituitary refractoriness was slowly overcome after cessation of the treatment, continuous infusion resulted in a more drastic desensitization of the releasing response of both gonadotropins than did pulsatile administration. It is suggested that effective desensitization of the pituitary gonadotropin response is elicited by continuous administration of leuprolide and, therefore, the vaginal administration resulting in prolonged serum levels of the analog would be favorable as a self-administration method for medical treatments by using the antagonistic activity such as anti-tumor therapy and reproduction control.

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