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Immunoreactivity for Gonadotropin-Releasing Hormone in Microdialyzed Perfusates of Bovine Mature Follicles *In Vitro*

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Abstract. The presence of gonadotropin-releasing hormone (GnRH)-like protein has been previously demonstrated in the bovine follicle. This GnRH-like protein was purified and concluded to be histone H2A. However, neither GnRH peptide nor specific GnRH-immunoreactivity (GnRH-IR) has been demonstrated in the bovine follicle so far. Thus, this study focused on the detection of specific GnRH-IR using the second-antibody enzyme immunoassay in isolated bovine mature follicles, and on an examination of the direct effect of luteinizing hormone (LH), endothelin-1 (ET-1), and cytokines on the GnRH-IR using an *in vitro* microdialysis system (MDS). We further examined a cross-reactivity of the GnRH antibody with bovine histone H2A. GnRH-IR was detected in microdialyzed perfusate from isolated bovine mature follicles at 4.40 ± 0.35 pg/ml (mean ± SEM). Bovine histone H2A showed no GnRH-IR at all. Heat treatment of the extract (100 C for 10 min) did not affect the GnRH-IR. Single infusion of LH, ET-1, or cytokines into the MDS did not affect the GnRH-IR. However, infusion of ET-1 after LH exposure increased the GnRH-IR. These results demonstrate the presence of specific GnRH-IR, that is different from histone H2A, in microdialyzed perfusate of isolated bovine mature follicles *in vitro*, suggesting that the GnRH-IR may reflect a role of GnRH-like peptide or some peptide structurally similar to GnRH in the local regulation of mature follicles.

Key words: GnRH, Immunoreactivity, Microdialysis, Follicle, Cow.

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Gonadotropin-releasing hormone (GnRH) is a neurodecapeptide secreted from the hypothalamus that stimulates the pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The fact that the GnRH concentration is very low in the systemic circulation and the fact that this peptide is rapidly degraded in the pituitary, brain, kidney, liver and serum suggest that hypothalamic GnRH can not be a physiological ligand of ovarian receptors. Previous studies have shown that exogenous GnRH and GnRH analogues have a direct inhibitory or stimulatory effect on the rat ovary [1–3] and bovine granulosa cells in culture [4, 5].

The presence of GnRH-like protein, GnRH mRNA, specific high-affinity binding sites for GnRH, and GnRH receptor mRNA have been demonstrated in the rat ovary [1, 6–11]. The bovine granulosa cells also contain higher concentrations of GnRH-like protein than other ovarian and extraovarian tissues [12]. This bovine GnRH-like protein was purified and concluded to be histone H2A [13]. However, the binding activities of

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GnRH-like proteins have been assessed by their binding to rat ovarian membranes by a radioreceptor assay (RRA) for GnRH [6, 12–14]. Neither GnRH peptide nor specific GnRH-immunoreactivity (GnRH-IR) in the rat, ovine, and bovine ovary have been demonstrated so far [6, 14].

In this study, we attempted to detect a specific GnRH-IR in the microdialyzed perfusates of isolated bovine mature follicles. Further, we examined cross-reactivity of the GnRH antibody with bovine histone H2A. The microdialysis capillary membrane was implanted in the theca layer where the integrity of follicular structures including granulosa cells, theca cells, blood vessels (endothelial cells), and follicular fluid was preserved [15]. The study was extended to observe the direct effects of LH, endothelin-1 (ET-1), tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) on the local concentration of GnRH-IR, i.e., the GnRH concentration measured in our immunoassay using the specific antibody for GnRH peptide, by utilizing the microdialysis system (MDS).

Materials and Methods

Collection of bovine mature follicles

Ovaries from Holstein cows containing a mature, presumably preovulatory follicle were collected within 20 minutes post-slaughter from a local slaughterhouse, and transported to the laboratory in sterile saline solution (0.9% NaCl) containing 100,000 IU penicillin and 100 mg streptomycin/l at 38 C. The preovulatory stage was defined by the presence of a Graafian follicle (between 1.5–2.0 cm in diameter) and a regressing corpus luteum in the ipsilateral or contralateral ovary [16]. The uterine characteristics (size, color, tonus, consistency, and mucus) were also considered. Furthermore, at the end of the experiment, follicular fluid from each follicle was collected, and the position of implantation of the MDS capillary membrane in the theca layer was confirmed with a dissecting microscope after bisection of follicles. Only dominant follicles that had not yet been exposed to the endogenous LH-surge were used for the data analyses. This was based on hormone concentrations in the follicular fluid collected at the end of experiments as follows; estradiol-17β (E_2), progesterone (P) and prostaglandin E_2 (PGE_2) were 324 ± 21 ng/ml, 34.6 ± 5.3 ng/ml and 355 ± 43 pg/ml for the pre-LH surge group, and 296 ± 45 ng/ml, 58.6 ± 5.6 ng/ml and 6145 ± 1552 pg/ml (mean ± SEM) for the post-LH surge group. Thus, the follicles used in this study were identified by having a PGE_2 concentration in the follicular fluid of less than 600 pg/ml [15].

MDS in vitro

The MDS for bovine mature follicles was described in detail previously [15]. Briefly, each follicle was dissected from surrounding stromal tissue, and four capillary dialysis membranes (Fresenius SPS 900 Hollow Fibers, cut-off Mr 1000 kDa, 0.2 mm diameter, 5 mm long; Fresenius AG, St Wendel, Germany) per follicle were implanted. The four capillaries were assigned to control, LH, cytokines or ET-1, and LH+cytokine or LH+ET-1, thus each treatment was applied to all the follicles. The prepared follicles were then placed in organ culture chambers (modified 2070 Tube; Falcon, Franklin Lakes, NJ, USA). During incubation, follicles were perfused with Ringer's solution at a flow rate of 1.8 ml/h. After 2 h pre-perfusion, fractions of the perfusate were collected every 2 h up to 14 h. Collected samples were stored at −20 C until hormone determination.

Bovine LH (USDA-bLH-B-6), human ET-1 (Peptide Institute Inc. Osaka, Japan), recombinant human TNF α and recombinant human IL-1β (both donated by Dainippon Pharm. Co., Osaka, Japan) were diluted in Ringer's solution to obtain the required final concentrations of 5 μg/ml, 250 ng/ml, 100 ng/ml and 10 ng/ml, respectively. The doses were confirmed to affect the release of steroids, ET-1 and PGE_2 [15]. Particularly, we previously showed that infusion of LH clearly increased the release of E_2 in this system [15]. The transfer capacity of the membrane was estimated to be 1% for steroids and prostaglandins, and 0.1% for peptides and LH [17, 18], when it was determined as described earlier [19]. The solutions were then infused into the MDS for 2 h (LH between 4–6 h; ET-1, TNFα or IL-β between 8–10 h).

GnRH-IR extraction

After the diethyl ether extraction at pH 3.5 for the purpose of analyses of steroids and PG [15], the remaining Ringer's solution (3 ml) was used

for extraction of GnRH-IR. BSA was added to the samples to a final concentration of 1 mg/ml. The samples were then applied to a Sep-Pak C_{18} Cartridge (Waters, Millford, MA, USA) as described previously [18]. The eluted residue was evaporated and then dissolved in 200 μl assay buffer (42 mM Na_2HPO_4, 8 mM KH_2PO_4, 20 mM NaCl, 4.8 mM EDTA, 0.05% BSA, pH 7.5) for peptide EIA.

GnRH-IR determination

The concentrations of GnRH-IR were determined in duplicate by second-antibody EIA using 96-well ELISA plates (Corning, New York, NY, U.S.A.). The GnRH EIA was identical to the ET-1 and oxytocin EIAs described previously [18]. We used the rabbit polyclonal antibody for GnRH (1:800 000) kindly supplied from Dr. Y. Hasegawa, Kitasato University, Towada, and biotinyl-GnRH (1:30 000) as a tracer. The standard curve ranged from 2 to 1000 pg/ml, and the ED_{50} of the assay was 90–130 pg/ml (Fig. 1). The intra- and interassay coefficients of variance (CVs) were 5.8% and 10.6%, respectively. The anti-GnRH antibody did not cross-react with oxytocin, synthetic TRH, or rat pituitary hormones (LH, FSH, and PRL) [20], or even with ET-1, angiotensin II, TNFα or IL-1β (<0.1%).

To examine the cross-reaction of bovine histone H2A, the potential bovine GnRH-like protein purified from bovine ovary [13], with the antibody against GnRH, bovine histone H2A (type VI-S from calf thymus; Sigma, St Louis, MO, USA) was tested by GnRH EIA described above.

Statistical analysis

The mean GnRH-IR concentrations in the first 2 perfusates (first 4 h perfusion with Ringer's solution only) were used to calculate the individual baselines, because of a large variation in the absolute amount of hormones released into each of the MDS lines implanted in the different follicles. GnRH-IR concentrations in the perfusate fractions were expressed as a percentage of the baseline levels. The effects of the infused substances (LH, ET-1, TNFα and IL-1β on the concentration of GnRH-IR were tested by Fisher's PLSD for the comparison among several treatment groups during the same period. The absolute concentrations of the GnRH-IR in the MDS fractions (mean ± SEM) are given in the figure legends.

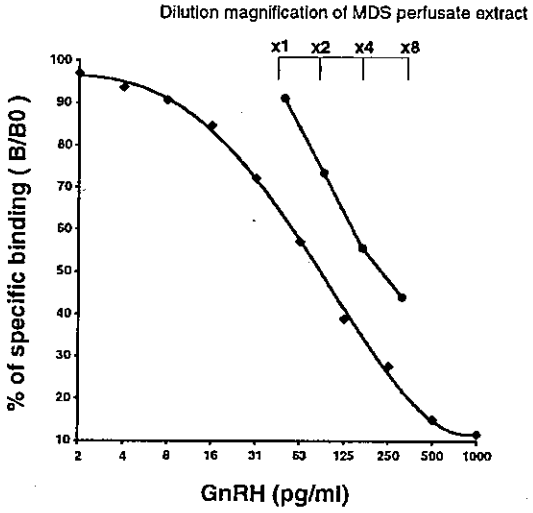

Fig. 1. Competitive binding of GnRH (◆—◆) and a perfusate extract (●—●) with a biotin-labelled GnRH in a second antibody enzyme immunoassay. (B/B_0: ratio of the amount specifically bound to the amount bound specifically at a zero dose.)

Results

The GnRH-IR was detected in the microdialyzed perfusates from isolated bovine mature follicles (4.40 ± 0.35 pg/ml, mean ± SEM). A serially diluted extract of the perfusate closely paralleled the competitive curve of GnRH binding (Fig. 1). Either the extract of BSA in Ringer's solution (10 mg/ml) alone and bovine histone H2A (10^{-12}–10^{-5} M) showed no GnRH-IR in the EIA at all. Heat treatment of the extract (100 C for 10 min) did not affect the GnRH-IR as in the case of the synthetic GnRH (Table 1).

The four capillaries were assigned to control, LH, cytokines or ET-1, and LH+cytokine or LH+ET-1, thus each treatment was applied to all the follicles.

Table 1. Effect of heat treatment (100 C, 10 min) on the immunoreactivities of synthetic GnRH and the extract of MDS perfusate in the second antibody EIA (mean ± SEM; n=5).

	GnRH (pg/ml)	MDS perfusate (pg/ml)
Control	58.9 ± 1.9	58.6 ± 1.4
Heat-treated	51.3 ± 2.6	54.6 ± 4.9

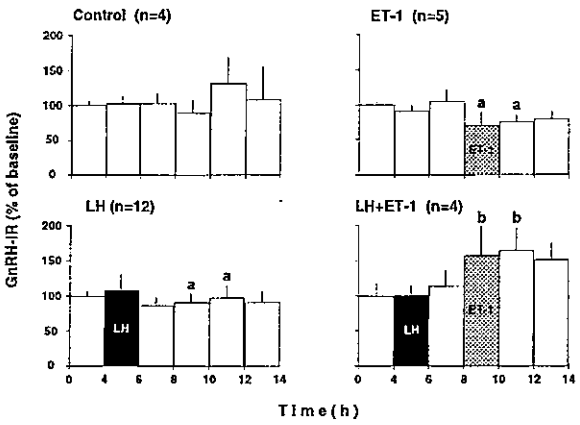

Fig. 2. Effect of LH (5 μg/ml) and ET-1 (250 ng/ml) infusion on GnRH-IR release from microdialyzed bovine mature follicles *in vitro*. Basically, the four capillary dialysis membranes were implanted per follicle, and thus all treatment could be applied in each follicle. The data of LH group were added from other follicles. Data are expressed as a percentage of the basal release (mean ± SEM). The baseline (100%) of GnRH-IR was 4.1 ± 0.4 pg/ml (n=25 lines; mean ± SEM). a vs b; $p<0.05$ during the same time period.

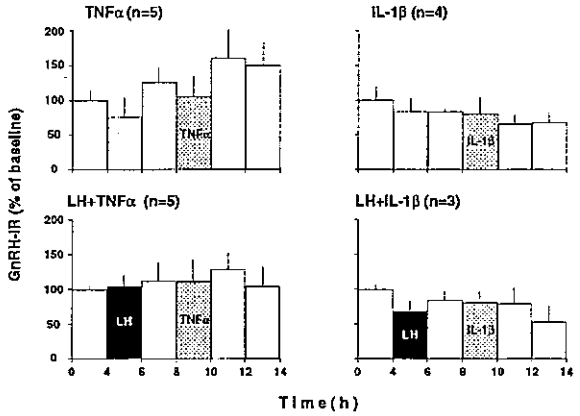

Fig. 3. Effect of LH (5 μg/ml), TNFα (100 ng/ml) and IL-1β (250 ng/ml) infusion on GnRH-IR release from microdialyzed bovine mature follicles *in vitro*. Basically, the four capillary dialysis membranes were implanted per follicle, and thus all treatment could be applied in each follicle. Data are expressed as a percentage of the basal release (mean ± SEM). The baseline (100%) of GnRH-IR was 4.9 ± 0.6 pg/ml (n=17 lines; mean ± SEM).

Single infusions of LH, ET-1, TNFα, and IL-1β into the MDS did not affect the concentration of GnRH-IR (Figs. 2 and 3). However, the infusion of ET-1 after LH exposure stimulated the GnRH-IR concentration until 2 h after ET-1 infusion compared with LH or ET-1 infusion alone ($p<0.05$; Fig. 2).

Discussion

This study clearly demonstrated the presence of specific GnRH-IR in microdialyzed perfusates obtained from the theca layer of bovine mature follicles *in vitro*. Earlier studies detected GnRH-like proteins in homogenates of rat [6], ovine, and bovine ovarian tissues [12, 14]. This bovine GnRH-like protein was purified and concluded to be histone H2A [13]. However, the anti-GnRH antibody in the present study did not cross-react with bovine histone H2A at all. This fact implies that the GnRH-IR in this study and the previously described bovine GnRH-like protein are different molecules. We attempted to detect the GnRH-IR in the media from bovine granulosa and theca cells culture, but both media showed no GnRH-IR (unpublished). In the present study, samples were obtained from bovine mature follicle by utilizing the MDS. This system allows the collection of substances directly from intercellular fluid in the theca layer where the cells maintain intact cell-to-cell contact. This raises the possibility that endothelial cells other than steroidogenic cells may secrete the GnRH-IR. Based on the GnRH-IR concentration in the perfusate (4.5 pg/ml) and the transfer capacity of the MDS capillary membrane (0.1%), the possible concentration in the intercellular fluid could be calculated around 4–5 ng/ml. This range is much higher than the range in peripheral plasma of cyclic cows (2–3 pg/ml in our EIA system; unpublished). Moreover, the antibody did not crossreact with other peptides released from the follicle. The peptide concentrations in the perfusates determined in our laboratory are 6.0 pg/ml for ET-1 [15], 45 pg/ml for angiotensin II and 15 pg/ml for atrial natriuretic peptide (ANP) [21], respectively. Therefore, it is unlikely that the present GnRH-IR is due to cross-reactivities with other local peptides with the GnRH antibody. The fact that the heat treatment of the extract (100 C for 10 min) did not affect the GnRH-IR suggests that the activity might be derived from unknown small peptides. In this context, the

possibility exists that the GnRH antibody reacted with peptides other than GnRH that possess amino sequence that are similar to, but different from, that of GnRH, hence the GnRH antibody recognized it well enough to react with it. The source of GnRH-IR and its molecular characteristics should be clarified in further studies.

GnRH-like protein, GnRH mRNA, specific high-affinity binding sites for GnRH, and GnRH receptor mRNA have been demonstrated to be present in rat ovarian tissues [1, 6–11]. A previous study reported that the expression of GnRH receptor mRNA in the rat ovary varies during the reproductive cycle. This expression was particularly high in the atretic and the preovulatory follicles [11]. These findings suggest that GnRH-like protein may play a role in the induction of follicular atresia and/or ovulation.

GnRH and its analogues have various effects on the secretion of steroid hormone and oxytocin/vasopressin by bovine granulosa cells in culture [4, 5]. However, the expression of GnRH receptor mRNA and the binding sites for GnRH have not yet been demonstrated in the bovine ovary [9, 22]. Recently, GnRH receptor mRNA was detected in the bovine mature cumulus-oocyte complex [23]. This suggests that the GnRH receptor is present in bovine follicular tissues, although it has not yet been found.

No changes in the local concentration of GnRH-IR were observed as the result of single infusions of LH, ET-1, and cytokines. Even if there is no significant change in GnRH-IR concentration, a chronic local secretion may be important for basic functions of the mature follicle, because of the relatively high concentration in the theca layer as described above. The infusion of ET-1 after LH exposure increased the concentration of GnRH-IR in the present study. This suggests that the LH-surge and local secretion of ET-1 may be responsible for increasing the GnRH-IR in the preovulatory follicle.

In conclusion, we have demonstrated a specific immunoreactivity for GnRH, that is different from histone H2A, in microdialyzed perfusate of isolated bovine mature follicles *in vitro*. Furthermore, the GnRH-IR may reflect a role of GnRH-like peptides or some peptide structurally similar to GnRH in the local regulation of mature follicles.

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