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Changes in Expression of Inhibin Subunits in the Cyclic Golden Hamster (*Mesocricetus auratus*) and the Regulation of Inhibin α Subunit Production by Luteinizing Hormone

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ABSTRACT—In the present study, changes in localization of each inhibin subunit in the ovary were investigated during the estrous cycle of the golden hamster. The effect of LH surge on changes in localization in inhibin α subunit in the ovary was also investigated.

Inhibin α subunit was localized in granulosa cells of various stages of follicles throughout the estrous cycle. Inhibin α subunit was also present in numerous interstitial cells on days 1 and 2 (day 1 = day of ovulation), but the number of positive interstitial cells was fewer on days 3 and almost disappeared on day 4 of the estrous cycle. Newly formed luteal cells were also positive for inhibin **α** subunit on days 1 and 2. On the other hand, positive reactions for inhibin βA and βB subunits were only present in the granulosa cells of healthy antral follicles. However, a positive reaction for inhibin βB subunit in peripheral mural granulosa cells disappeared on days 3 and 4 of the estrous cycle. Treatment with LHRH-AS at 1100 h on day 4 completely blocked the luteinizing hormone (LH) surge and ovulation, although relatively high concentrations of plasma follicle-stimulating hormone (FSH) were maintained throughout the experiment. There were few positive reactions for inhibin α subunit in theca and interstitial cells 24 hr after LHRH-AS injection. The effect of LHRH-AS treatment was blocked by a single injection of 10 IU human chorionic gonadotropin.

These results suggest that the major source of dimeric inhibin in the cyclic hamster was granulosa cells of healthy antral follicles. Different distribution pattern of inhibin βA from βB subunits in large antral follicles on days 3 and 4 of the estrous cycle suggests different secretion patterns of inhibin A from B on these days. Furthermore, the LH surge may be an important factor to induce production of inhibin α subunit in interstitial cells of the cyclic hamster.

Key words: inhibin, ovary, interstitial cell, LH, hamster

INTRODUCTION

Inhibin molecules are now well known as glycoprotein hormones secreted from gonads in many species; they are composed of a common α subunit and one of two similar, but distinct β subunits (βA and βB). They are designated as inhibin A or inhibin B based on the βA or βB subunit, respectively. Inhibin is also well known as a key regulatory factor of FSH secretion. We also demonstrated the importance of inhibin on FSH regulation in the female (Kishi *et al*., 1995, 1996, 1997a, 1999) and male (Kishi *et al*., 2000) hamsters.

Follicle-stimulating hormone (FSH) is believed to be essential for ovarian follicular development and maturation in female animals, and also stimulates inhibin production in granulosa cells (Henderson *et al*., 1984; Bicsak *et al*., 1986; Ying *et al*., 1987) and Sertoli cells (Steinberger, 1981; Le Gac and de Kretzer, 1982; Ultee-van Gessel *et al.* 1986; Ying *et al*., 1987). Luteinizing hormone (LH) also affects inhibin production in granulosa cells (Tsonis *et al*., 1987; Zhang *et al*., 1988; Hillier *et al*., 1991) and Leydig cells (Risbridger *et al*., 1989; Simpson *et al*., 1991). These reports

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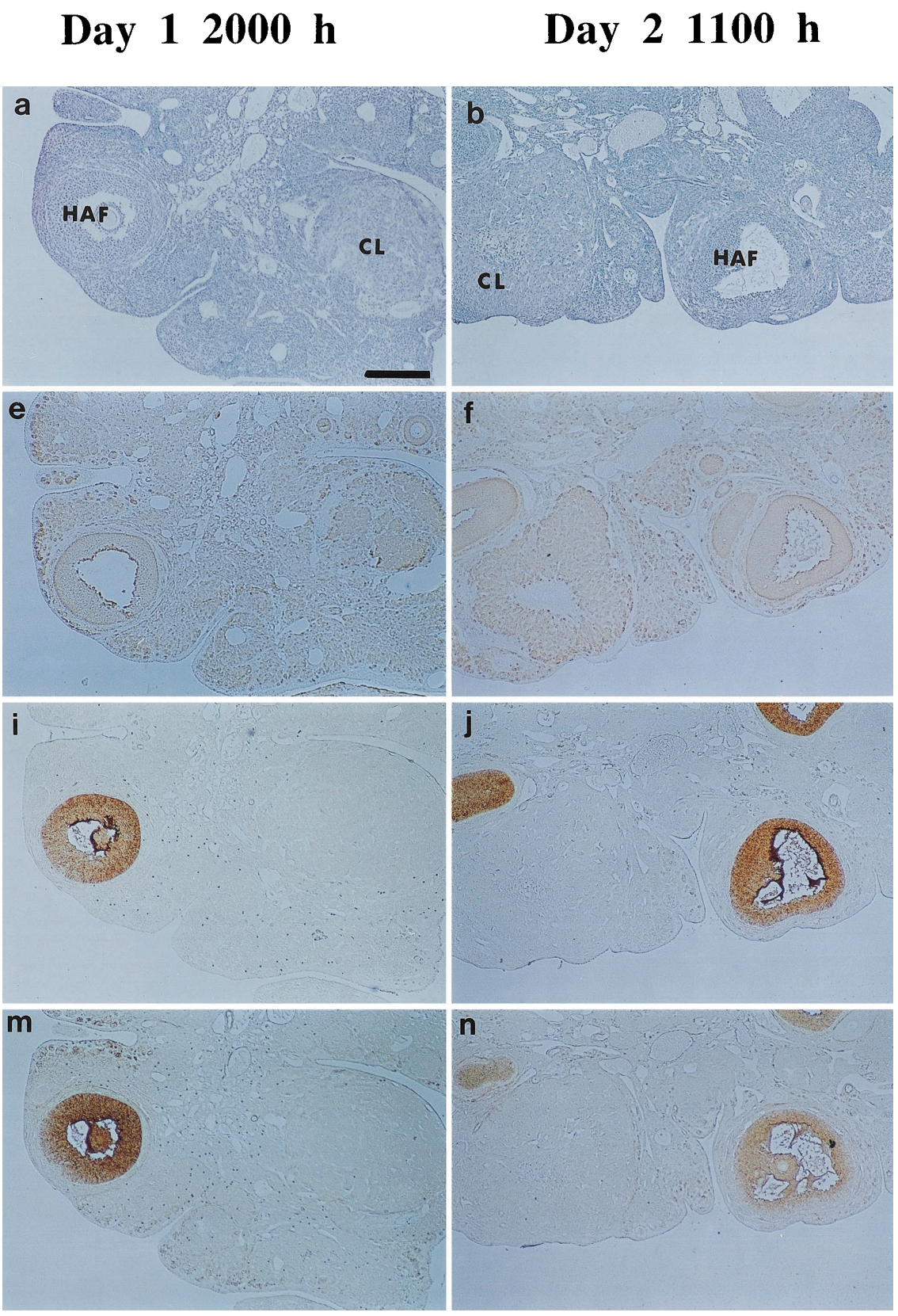
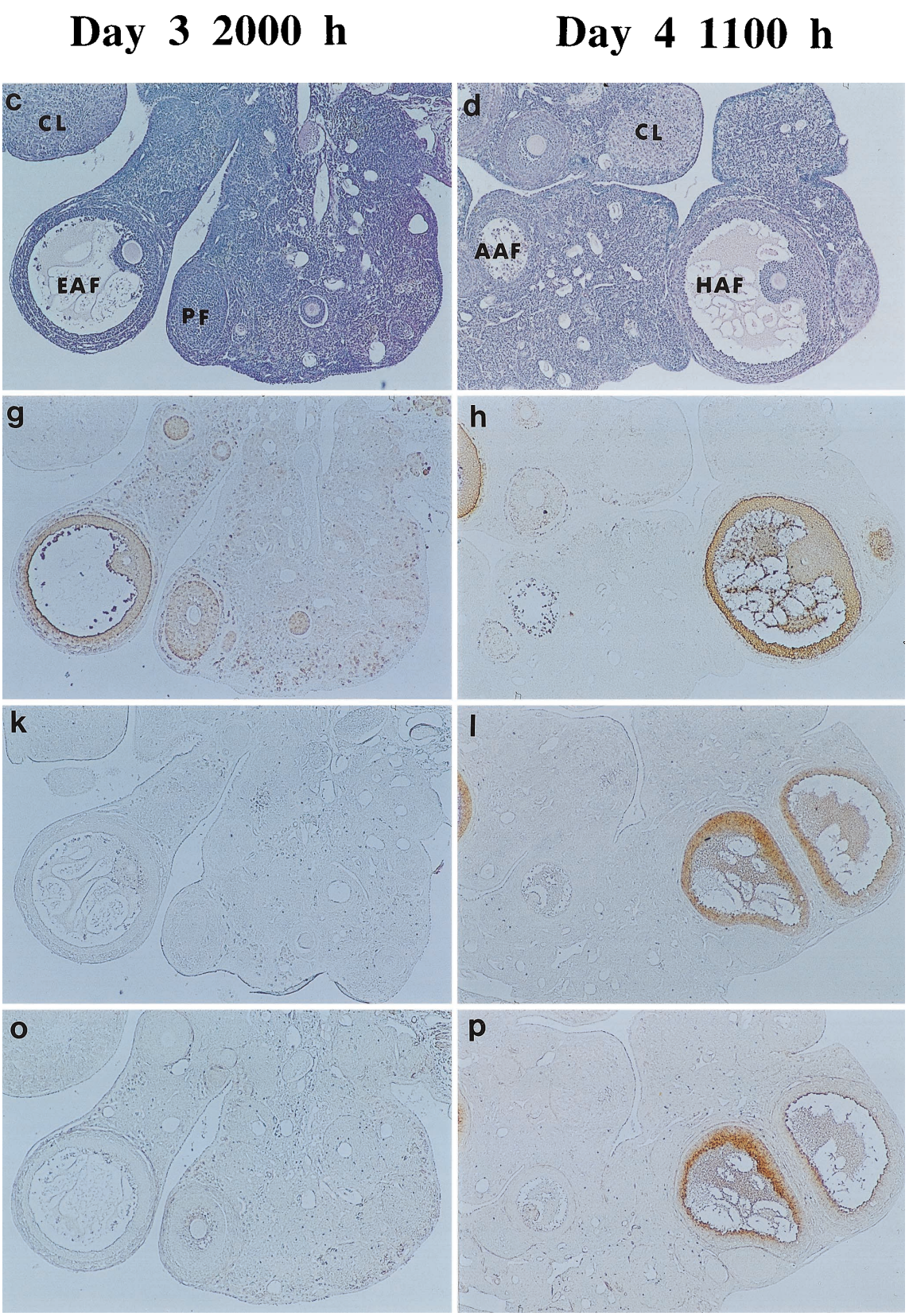


Figs. 1. Localization of each subunit of inhibin molecules in the ovary during the estrous cycle of the hamster. Left column (a, e, i, m), second column (b, f, j, n), third column (e, g, k, o) and fourth column (d, h, l, p) show the ovarian sections obtained at 2000 h on day 1, 1100 h on day 2, 2000 h on day 3 and 1100 h on day 4 of the estrous cycle in the hamsters. The staining of each section is as follows: a–d stained with hematoxylin and eosin; e–h stained with inhibin α subunit antiserum; i–l stained with inhibin βA subunit antiserum; and m-p stained with



inhibin βB subunit antiserum, respectively. Ovarian structures are shown as follows: HAF, healthy antral follicle; EAF, early atretic follicles; AAF, advanced atretic follicles; PF, preantral follicles; CL, corpus luteum. The solid bar in panel a represents 250 μm. All setions were photographed using the same magnification.

indicate that gonadotropins are major regulators of gonadal inhibin secretion in both male and female animals. The effects of gonadotropin on follicular development had been described in many species (Greenwald, 1994). We also reported that equine chorionic gonadotropin (eCG) treatment to intact cyclic hamsters causes superovulation and high plasma concentrations of immunoreactive (ir-) inhibin (Kishi *et al.,* 1997b). Treatment with antiserum against eCG to female hamsters, which had been hypophysectomized and treated with eCG 4-days before, induced follicular atresia and declining plasma concentrations of ir-inhibin (Otsuka *et al*., 1997). These results suggest that gonadotropins promote follicular development, and stimulate gonadal inhibin secretion in female hamsters as well as other species. Furthermore, We demonstrated cyclic changes in plasma concentrations of inhibin A, B and pro-α C in the hamster during the estrous cycle (Ohshima *et al*., 1999). This report showed several different changes among plasma concentrations of inhibin A, B and pro-α C during the estrous cycle of the hamster. Therefore, expression pattern of each subunit of inhibin in the ovary would change under the episodic changing gonadotropins secretion during the estrous cycle of hamsters.

We determined, therefore, changes in immunopositive reaction of three inhibin molecules (α, βA or βB subunit) in each ovarian cell type throughout the estrous cycle of the hamster. In the next study, we also determined the physiological role of LH surge on immuno-expression of inhibin in the ovary of cyclic hamsters.

MATERIALS AND METHODS

Animals

Adult female golden hamsters (*Mesocricetus auratus*) were maintained on a 14 L: 10 D cycle (lights on at 0500 h) with food and water provided *ad libitum*. The day of estrus (day 1) was determined by the presence of the characteristic vaginal discharge. Animals with at least two consecutive 4-day estrous cycles were used in the present study.

Immunohistochemical localizations of inhibin α, βA and βB during the estrous cycle

Ovaries were removed under light ether anesthesia from the cyclic hamsters at 1100 or 2000 h on every day of the estrous cycle. The ovaries were embedded in paraffin after fixation with methacarn and were sectioned at 6 μm. The immunostaining was performed as described previously (Otsuka *et al*., 1997). TNDK1 (Kishi *et al*., 2000) was used as a primary antiserum for the specific detection of inhibin α subunit, and rabbit anti-cyclic inhibin βA (81-113)-NH_2 (Code #305-24-D: Vaughan *et al*., 1989) and rabbit anti-cyclic inhibin βB (80-112)-NH_2 (Code #305-25-D: Vaughan *et al*., 1989) (kindly provided by Dr. W. Vale, Salk Institute for Bilogical Studies, La Jolla, CA, USA) were used as primary antisera for the specific detection of inhibin βA and βB subunits, respectively.

Immunoneutralization of circulating LHRH

The antiserum against LHRH (LHRH-AS) used in the present study was the same antiserum previously described by Matsuzono *et al* (1986). Adult cyclic hamsters were given a single i.v. injection of 200 μl LHRH-AS at 1100 h on day 4 of the estrous cycle. At 1700 h on day 4, 10 IU human chorionic gonadotropin (hCG; Sankyo Zoki Ltd., Tokyo, Japan) or saline was treated (iv) in each group of animals, which had been given LHRH-AS as above. Groups of animals were decapitated at various hours after LHRH-AS treatment, and trunk blood was collected into each heparinized centrifuge tube. Plasma samples were separated and stored at –20°C until assayed for FSH and LH. Ovaries were also collected from the animals 24 hr after treatment with LHRH-AS, and were studied for localization of inhibin α subunit as compared with the intact animals.

Radioimmunoassays (RIAs)

Concentrations of each hormone in plasma were determined by specific radioimmunoassays. Plasma concentrations of FSH and LH were measured using NIDDK RIA kits for rat FSH and LH as described previously (Bast and Greenwald, 1974). Iodinated preparations were rat FSH-I-8 and LH-I-9. The antisera used were anti-rat FSH-S-11 and LH-S-9. Results were expressed in terms of NIDDK rat FSH-RP-2 and LH-RP-2. The intra- and inter-assay coefficients of variation were 4.4 and 14.6% for FSH and 8.9 and 6.7% for LH, respectively.

Statistics

All data were expressed as means±SEM of five animals. Significance of the difference between two means was tested by Student's *t*-test or Chochran-Cox test; probability less than 0.05 were considered statistically significant.

RESULTS

Immunohistochemical localizations of inhibin α, βA and βB subunits in the ovary of normal cyclic hamsters (Fig. 1 and Table 1)

Immunopositive reactions for inhibin α subunit were found in granulosa cells of various stages of follicles throughout the estrous cycle of the hamster, even if the follicles were entering atresia (Fig. 1e~h; Table 1). Numerous interstitial cells also showed immunopositive reaction for inhibin α subunit on days 1 (Fig. 1e) and 2 (Fig. 1f), but the number of positive reaction cells evidently decreased on day 3 (Fig. 1g) and almost disappeared on day 4 (Fig. 1h). Although immunopositive inhibin α subunit was found in luteal cells on days 1 (Fig. 1e) and day 2 (Fig. 1f) of the estrous cycle, the positive reaction disappeared on days 3 and 4.

Throughout the estrous cycle, immunopositive localization of inhibin βA subunit was present in granulosa cells of healthy antral follicles, but not preantral and atretic follicles (Fig. 1i~l). Immunopositive inhibin βB subunit was also present in granulosa cells of only healthy antral follicles as same as inhibin βA subunit (Fig. 1m~p). Compared with that of the α subunit, the reduction of immunopositive reactions of inhibin βA and βB subunits occurred at earlier stages of atresia (Fig.1k, o). The distribution of immunopositive inhibin βB, but not βA, was not uniformed in each follicle, especially on days 3 and 4. Mural granulosa cells rounding on the antral cavity of each antral follicle had an intense positive reaction to inhibin ßB; the immunopositive reaction was reduced and disappeared in the mural granulosa cells located in the periphery of each antral follicle.

Table 1 Immunohistochemical staining of hinibin subunits in ovarians of cyclic golden hamster

Inhibin subunits and stage of the estrous cysle	α				βA				βB			
	day 1	day 2	day 3	day 4	day 1	day 2	day 3	day 4	day 1	day 2	day 3	day 4
Granulosa cells												
preantral follicles	+	+	+	+	−	−	−	−	−	−	−	−
healthy antral follicles	+	+	+	+	+	+	+	+	+	+	+	+
early atretic follicles	+	+	+	+	−	−	−	−	−	−	−	−
advanced atretic follicles	+	+	+	+	−	−	−	−	−	−	−	−
Interstitial cells	++	++	+	−	−	−	−	−	−	−	−	−
Corpus luteum	+	+	−	−	−	−	−	−	−	−	−	−

The immunohistcheical staining was determined as positive (+), strong positive (++) or negative (–).

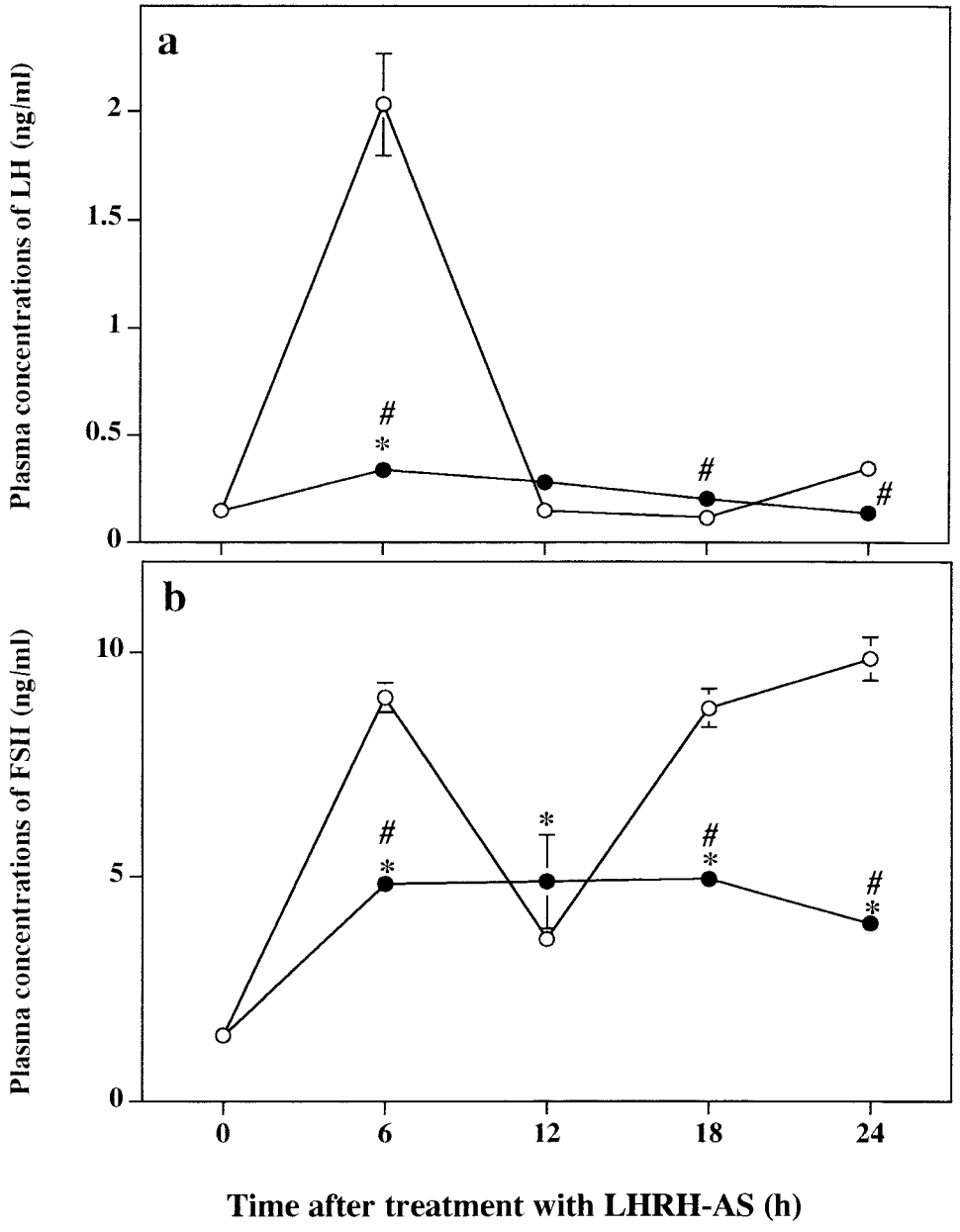
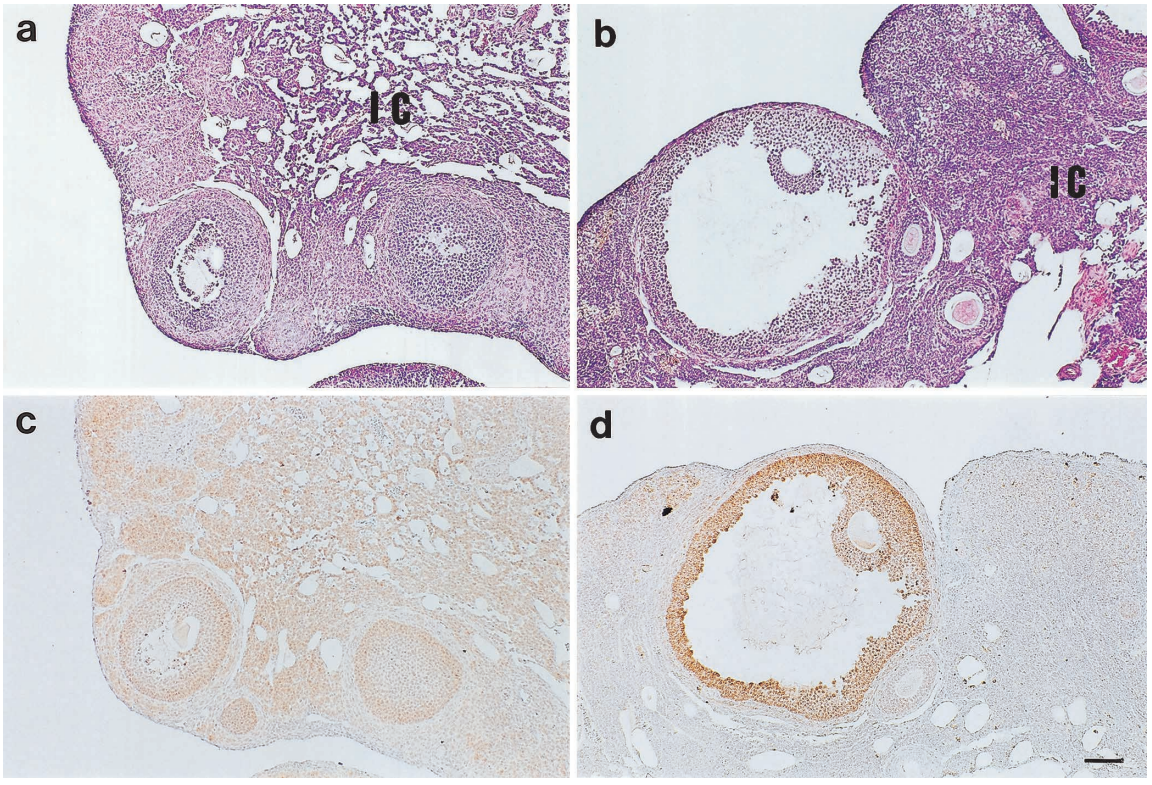


Fig. 2. Changes in plasma concentrations of (a) LH and (b) FSH of normal estrous cycle (○) and that after treatment with luteinizing hormone releasing hormone antiserum (LHRH-AS) at 1100 h on day 4 of the estrous cycle (●). Each value represents the mean±S.E.M. of five animals. Values with asterisc represent statistically ($p<0.05$) different from the values at the time of LHRH-AS injection (0h). #, significantly different from the values at the time matched- control ($p<0.05$).



Figs. 3. Changes in localization of inhibin α subunit in the ovary 24 hr after treatment with antiserum against LHRH (LHRH-AS). The animals were given 10 IU hCG (a, c) or saline (b, d) 6 hr after the LHRH-AS treatment. (a) and (b) showed the section by stained with hematoxylin eosin and (c) and (d) showed the section by stained with inhibin α subunit antiserum. IC; interstitial cells. The solid bar in panel (d) represents 100 μm. All sections were photographed using the same magnification.

Effects of treatment with LHRH-AS

Plasma concentrations of gonadotropins (Fig. 2)

After treatment with LHRH-AS at 1100 h on day 4, the characteristic vaginal discharge and spontaneous ovulation did not occur in the animal 24 hr after the treatment. The preovulatory LH surge was completely blocked by treatment with LHRH-AS and basal levels of LH were observed throughout the experimental period. Plasma concentrations of FSH in the LHRH-AS treated animals were significantly low as compared with that of normal cyclic animals, whereas the levels were significantly high as compared with the value before treatment (0 h). Relatively high levels of the basal FSH were maintained throughout the experiment.

Immunohistochemical study of inhibin α subunit (Figs. 3)

In the both groups of animals, a positive reaction for inhibin α subunit was observed in granulosa cells of various sized follicles 24 hr after treatment with LHRH-AS as well as in the ovary obtained from intact cyclic animals at 1100 h on day 1. In the ovary of animals treated with LHRH-AS and saline, however, a positive reaction for inhibin α subunit was not found in interstitial cells unlike those in the intact animals. On the other hand, clear immunopositive reaction for inhibin α subunit was found in interstitial cells of the animals which 10 IU hCG was given 6 hr after LHRH-AS treatment (it is the time when expected LH surge occurred in intact animal). Immunopositive reaction for inhibin α subunit in the LHRH-AS plus hCG treated animals was the same as that in intact animals at 1100 h on day 1.

DISCUSSION

The present results clearly indicate that inhibin α subunit was localized in interstitial cells, as well as granulosa cells of the various stages of follicles, on days 1 and 2 of the estrous cycle of the hamster, and the immunostained cells disappeared on days 4. These findings suggest that ovarian interstitial cells of the hamster produce α subunit of inhibin. Meunier *et al.* (1988) also described that mRNA of inhibin α subunit was expressed in interstitial and theca cells in the ovary of cyclic rat. Treatment with LHRH-AS, in the present study, completely blocked the LH surge and ovulation, but only a slight effect was observed in plasma concentrations of FSH. The positive reaction of inhibin α subunit in most of all interstitial cells was disappeared in those animals unlike in intact animals. This effect of LHRH-AS on interstitial cells was recovered by hCG treatment instead of saline. Furthermore, in the hamster, Oxberry and Greenwald (1982) demonstrated that interstitial cells have LH, but not FSH receptor. Together with these findings suggest that high levels of LH, such as the LH surge, may stimulate the expression of inhibin α subunit in the interstitial cells. Many investigators

have focussed on the regulation of inhibin molecules in granulosa cells by FSH (Turner *et al*., 1989; LaPolt *et al*., 1990; Aloi *et al*., 1995; Tekmal *et al*., 1996). As far as we know, the present report would be the first demonstration that LH could induce production of inhibin α subunit in interstitial cells.

The present results showed that inhibin α subunit was also present in the granulosa cells of various (healthy preantral, antral or early atretic) stages of follicles as described in our previous paper using hamsters (Otsuka *et al*., 1997). On the other hand, inhibin βA and βB subunits were localized only in the granulosa cells of antral follicles during the estrous cycle in the hamster. These results are agreed with a previous rat study (Uilenbroek *et al*. 1998). The intensity of immunopositive reactions of inhibin βA and βB subunits in atretic follicles disappeared more rapidly than that of inhibin α subunit. These findings suggest that dimeric, probably bioactive, inhibin is mainly secreted from healthy antral follicles. Furthermore, in the present study, intensity of immunpositive reaction of inhibin βB subunit in granulosa cells which are in peripheral zone was less or disappeared on days 3 and 4, although these follicles looked like healthy Graafian follicles. This is also demonstrated by a previous study using rats (Uilenbroek *et al*., 1998). These results suggest that there are some physiological differences between inner and outer layer of granulosa cells in the follicles in the hamster as well as rat. Collectively, the expression of inhibin βB subunits seems to be more related to follicular maturation or atresia than that of α and βA subunits.

The temporal changes in the distribution of immunopositively reactive inhibin βB subunit, as described above, might be related to changing plasma concentrations of inhibin B during the estrous cycle of the hamster. The present results show that the expression of inhibin βB subunit in peripheral mural granulosa cells disappeared until 2000 h on day 3. On the other hand, Ohshima *et al*. (1999) reported that plasma concentrations of inhibin B started to decline around this time. Therefore, the decline of immunostaining intensity of inhibin βB subunit in the peripheral granulosa cells on days 3 and 4 would be responsible for the reducing plasma concentrations of inhibin B in the later half of the estrous cycle of the hamster.

We also observed the immunopositive reaction of inhibin α, but not βA and βB subunits in luteal cells on days 1 and 2. These results suggest that newly luteinized cells may secrete inhibin α subunit in the hamster. In a previous rat study, only newly formed corpora lutea also exhibited a positive reaction for inhibin α subunit (Meunier *et al*., 1988). Therefore, production of inhibin α subunit would be sustained longer through their cellular differentiation, from granulosa to luteal cells, than those of inhibin βA and βB subunits.

A previous report by Ogawa *et al*. (1991) demonstrated that immunopositive localization of inhibin βA subunit was found in oocytes in the adult rats. On the other hand, we did not found the localization of inihibin βA subunit in any oocyte in the present study. Furthermore, Meunier *et al*. (1988) also reported that neither inhibin βA subunit mRNA nor immunopositive reaction was found in oocytes, using cyclic rats. We do not know the reason why different results come from. One of the possibilities is that using different primary antibodies to inhibin βA subunit might be responsible for these different results.

In conclusion, LH induces inhibin α subunit production in interstitial cells in the cyclic hamster. Inhibin α subunit was localized in granulosa cells throughout their life span. Furthermore, expression of inhibin β subunits, especially βB subunit, may be related to the maturation or differentiation of granulosa cells of antral follicles.

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