Secretory pattern of inhibin A, inhibin B and inhibin pro- α C during induced follicular atresia and subsequent follicular development in the golden hamster (*Mesocricetus auratus*)

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Abstract

The changes in plasma concentrations of inhibins A, B and pro- α C were determined in the cyclic golden hamster during follicular atresia induced with antiserum against luteinizing hormone releasing hormone (LHRH-AS) at 1100 h on day 4 (day 1 = day of ovulation). Follicular status in the ovary was also studied by determining the number of follicles ovulating in response to human chorionic gonadotrophin (hCG) injection. The time-courses of changes in plasma concentrations of inhibins A, B and pro- α C were different from each other during induced follicular atresia and subsequent follicular development. Plasma concentrations of inhibin A decreased to 58.6% of initial values by 24 h after LHRH-AS treatment, and then remained relatively low until at least 60 h later. Plasma concentrations of inhibin B decreased to 64.2% of the initial values by 18 h after LHRH-AS treatment and remained at basal values for 36 h, but increased abruptly to greater than initial values at 42 h after the treatment. Plasma concentrations of inhibin pro- α C increased at

6 and 12 h, decreased suddenly to 21.9% of the initial values by 24 h after LHRH-AS treatment, and then gradually increased until 60 h after LHRH-AS. The number of follicles responding to hCG decreased gradually between 0 and 30 h after LHRH-AS, when no ovulations were observed, and then gradually increased until 60 h. The changes in follicular ovulatory responses to hCG correlated with the plasma profile of inhibin A throughout the experiment. These results suggest that inhibin A is mainly secreted by large antral follicles. In contrast, during the subsequent follicular development, the plasma concentration of inhibin B increased earlier than that of inhibin A. These results suggest that inhibin B is secreted by small and large antral follicles. Plasma concentrations of inhibin pro- α C were high at a time when plasma concentrations of oestradiol-17 β had already decreased, indicating that inhibin pro- α C is secreted not only from healthy follicles but also from early atretic antral follicles.

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Introduction

Inhibin is a glycoprotein hormone secreted from granulosa cells of the ovarian follicles in female animals and is a key factor for determining species-specific ovulation rates through the regulation of follicle-stimulating hormone (FSH) secretion (Kishi *et al.* 1996, 1997*a,b*, Taya & Watanabe 1999). We have previously shown that inhibin bioactivity in ovarian venous plasma decreased gradually during the process of follicular atresia, whereas concentrations of oestradiol-17 β declined quickly after hypophysectomy at 1100 h on the day of pro-oestrus in the rat (Kaneko *et al.* 1987). We have also previously demonstrated that granulosa cells continue to secrete immuno-

reactive inhibin during the process of follicular atresia in the hamster (Otsuka *et al.* 1997). However, the time-courses of plasma concentrations of inhibin A and inhibin B during induced follicular atresia are not known. Recently, a new enzyme-linked immunosorbent assay (ELISA) has been developed (Groome & Lawrence 1991, Groome *et al.* 1995, 1996) that has been adapted to the specific measurement of different circulating forms of inhibins. In another previous study, we investigated the pattern of plasma concentrations of inhibins A, B and pro- α C during the normal oestrous cycle of the female golden hamster (Ohshima *et al.* 1999) and noted significant negative relationships between plasma FSH and both dimeric inhibins from day 1 to day 3, together

with significant positive relationships between plasma oestradiol-17 β and inhibin A or inhibin pro- α C, but no significant relationship between plasma oestradiol-17 β and inhibin B throughout the oestrous cycle.

In the present study, relationships between circulating inhibins (A, B and pro- α C) and ovarian follicular activity were studied during induced follicular atresia and subsequent follicular development induced by immunoneutralization of luteinizing hormone (LH) releasing hormone (LHRH). Relationships between circulating FSH and each inhibin molecule were also investigated in this present model.

Materials and Methods

Animals

Adult female golden hamsters (*Mesocricetus auratus*) were maintained under controlled temperature and lighting (lights on from 0500 h to 1900 h). Food and water were available *ad libitum*. The 4-day oestrous cycle was determined by the presence of a characteristic vaginal discharge on the morning of the day of ovulation, which was designated day 1 of the cycle. Hamsters with at least two consecutive 4-day oestrous cycles were used in this study.

Antiserum against LHRH

LHRH antiserum (LHRH-AS) was prepared as described previously (Matsuzono *et al.* 1986). In brief, a castrated goat was immunized against LHRH (NIDDK, Bethesda, MD, USA) conjugated to bovine serum albumin (BSA). The control serum was obtained from a castrated goat immunized against BSA alone.

Experimental design

LHRH-AS (0.2 ml) or control serum (0.2 ml) was injected into the jugular vein with the animal under light ether anaesthesia, at 1100 h on day 4 of the cycle. (In previous studies (Matsuzono *et al.* 1986), this dose of LHRH-AS completely blocked the preovulatory LH surge and subsequent ovulation, and also induced follicular atresia in the cyclic rat.) Animals were killed, by decapitation, at 6-h intervals from 1100 h on day 4 (just before the injection) to 60 h after the treatment, and the ovaries were removed for histological study. Trunk blood was collected into heparinized centrifuge tubes. Blood samples were centrifuged immediately after sampling, and plasma was separated and stored at -20°C until required for hormonal assay.

Radioimmunoassay of LH, FSH, progesterone and oestradiol-17 β

Concentrations of LH and FSH in plasma were measured using NIDDK RIA kits for rat LH and FSH as described

Figure 1 Changes in plasma concentrations of (a) LH, (b) FSH, (c) progesterone and (d) oestradiol-17 β after treatment with control serum (○) and LHRH-AS treatment (●) at 1100 h on day 4 of the oestrous cycle. Each value represents the mean $\pm$ S.E.M. of five observations. Significant differences ($P < 0.05$): *compared with the respective control; #compared with the value at 1100 h on day 4 of the oestrous cycle.

previously (Bast & Greenwald 1974). Iodinated preparations were rat LH-I-5 and FSH-I-5. The antisera used were anti-rat LH-S-9 and anti-rat FSH-S-11. The intra- and interassay coefficients of variation were 8.9% and 6.7% for LH and 4.4% and 14.6% for FSH respectively.

Figure 2 Changes in plasma concentrations of (a) inhibin A, (b) inhibin B and (c) inhibin pro- α C after treatment with control serum (○) and LHRH-AS treatment (●) at 1100 h on day 4 of the oestrous cycle. Each value represents the mean $\pm$ S.E.M. of five observations. Significant differences ($P < 0.05$): *compared with the respective control; #compared with the value at 1100 h on day 4 of the oestrous cycle.

Concentrations of progesterone and oestradiol-17 β in plasma were measured by double-antibody RIA systems using 125 I-labelled radioligands as described previously (Taya *et al.* 1985). Antisera against progesterone (GDN 337; Gibori *et al.* 1977) and oestradiol-17 β (GDN 244; Korenman *et al.* 1974) were kindly provided by Dr G D Niswender (Colorado State University, Fort Collins, CO, USA). The intra- and interassay coefficients of variation were 6.3 and 15.4% for progesterone and 3.7% and 6.2% for oestradiol-17 β respectively.

ELISA of inhibins A, B and pro- α C

Inhibin A, inhibin B and inhibin pro- α C assays for human were purchased from Serotec (Oxford, Oxon, UK). The characteristics of these dimer-specific assays for hamster

have been described previously (Ohshima *et al.* 1999). Our previous studies indicated that it is possible to measure the concentration of inhibins A, B and pro- α C in peripheral plasma of the female golden hamster using the human ELISA system. Amounts of inhibins A and B were expressed in terms of recombinant human inhibin A (Groome & Lawrence 1991) and recombinant human inhibin B (Groome *et al.* 1996) respectively. The amount of inhibin pro- α C was expressed in terms of the purified inhibin pro- α C from human follicular fluid (Groome *et al.* 1995). The intra- and interassay coefficients of variation are less than 10% for inhibin A, less than 7% for inhibin B and less than 20% for inhibin pro- α C. The sensitivities of the assays for inhibins A, B and pro- α C were 0.74 ± 0.10 pg/well ($n=21$), 1.64 ± 0.14 pg/well ($n=19$) and 0.19 ± 0.03 pg/well ($n=11$) respectively.

Ovarian follicular profiles during induced follicular atresia

To determine the number of large healthy antral follicles in the ovary after induction of follicular atresia, groups of animals were given 10 IU human chorionic gonadotrophin (hCG) (2200 IU/mg; Sankyo Zoki Co., Tokyo, Japan) dissolved in 0.2 ml saline, injected into the jugular vein with the animal under ether anaesthesia, at 6-h intervals after treatment with LHRH-AS (*n*=5). Animals were killed by decapitation at 18–20 h after hCG injection, and then the oviducts were examined for oocytes.

Ovarian histology

Ovaries were fixed in methacarn fixative for 8 h. After fixation, they were dehydrated in alcohol baths, ending in xylene, embedded in paraffin, sectioned serially at 6 μm and stained with haematoxylin and eosin for observation of morphological changes during follicular atresia.

Statistics

All data were expressed as means ± S.E.M. One-way ANOVA was performed. When variance between two means was uniform, Student's *t*-test was used, but when it was not uniform, the Cochran-Cox test was used. A value of *P*<0.05 was considered statistically significant.

Results

Plasma concentrations of LH, FSH, progesterone and oestradiol-17β after LHRH-AS treatment

The preovulatory LH surge noted at 1700 h on day 4 in the control animals was completely blocked by the administration of LHRH-AS (Fig. 1a). Plasma concentrations of LH were low throughout the period studied.

Plasma concentrations of FSH increased and reached a plateau within 6 h after LHRH-AS treatment (Fig. 1b). In the control animals, plateau concentrations were significantly lower than the peak preovulatory values, and the second surge of FSH occurred at 1700 h on day 4. In treated animals, plasma concentrations of FSH remained relatively high until 36 h and then gradually decreased until 60 h after the treatment.

The increase in circulating progesterone noted after the preovulatory LH surge in the control animals did not occur in the animals treated with LHRH-AS. In the latter group, plasma concentrations of progesterone remained low until 24 h, increased abruptly at 30 h, declined gradually until 48 h, and then remained low until 60 h after the treatment (Fig. 1c).

The profile of oestradiol-17β concentrations after LHRH-AS treatment was similar to that in the control animals. Plasma concentrations of oestradiol-17β reached a peak at 6 h, decreased abruptly to low values within 12 h after the LHRH-AS treatment, remained low for 36 h, and exhibited a gradual increase thereafter (Fig. 1d).

Table 1 Effects of hCG on hamsters treated with LHRH-AS. Hamsters were injected with LHRH-AS at 1100 h on day 4 and with 10 IU hCG at various times. The values are mean with or without S.E.M. of tubal oocytes per ovulating animal

Time of hCG injection (h after LHRH-AS)	No. of oocytes/ ovulating hamster	Hamsters ovulating/ hamsters examined (No. (%))
0	16.0 ± 1.1	5/5 (100)
6	16.3 ± 4.5	3/3 (100)
12	13.8 ± 1.3	5/5 (100)
18	11.6 ± 0.2*	5/5 (100)
24	9 (13, 5)	2/5 (40)
30	0	0/5 (0)
36	6 (11, 1)	2/5 (40)
42	11.3 ± 2.2	3/5 (60)
48	5.3 ± 3.4*	3/5 (60)
54	6.8 ± 1.5*	5/5 (100)
60	9.8 ± 1.5	5/5 (100)

*Statistically (*P*<0.05) different from the values at the time of LHRH-AS injection (0 h).

Plasma concentrations of inhibins after LHRH-AS treatment

Different concentration profiles were observed for circulating inhibins A, B and pro-αC after a single administration of LHRH-AS. The preovulatory inhibin A surge, usually noted at 1700 h on day 4 in the control animals, was completely blocked by LHRH-AS treatment. Plasma concentrations of inhibin A decreased to 58.6% of initial values by 24 h after LHRH-AS treatment and then remained low throughout the remaining period of study (Fig. 2a).

Plasma concentrations of inhibin B decreased to 64.2% of the initial value within 18 h and remained low until 36 h (Fig. 2b), after which they increased abruptly to greater than the initial values, 42 h after the LHRH-AS treatment. These greater concentrations were maintained throughout the remainder of the experimental period. Changes in plasma concentrations of inhibin B were negatively correlated with the changes in FSH (*r*=−0.516; *n*=55).

Plasma concentrations of inhibin pro-αC increased and remained high until 12 h after LHRH-AS treatment (Fig. 2c). Thereafter, they declined abruptly and reached a nadir (21.9% of initial values) at 24 h, followed by a gradual increase up to 60 h after LHRH-AS treatment.

Ovarian follicular profiles during induced follicular atresia

Table 1 gives details of the ovarian response to hCG in animals treated with LHRH-AS. The number of ovulations in response to hCG decreased significantly at 18 h after the LHRH-AS treatment as compared with that at time 0. No ovulatory response to hCG was noted at 30 h

Figure 3 Time-related morphological changes in antral follicles in the ovary during induced follicular atresia by treatment with LHRH-AS, in the golden hamster. Healthy antral follicles at 0 h (a), 42 h (d) and 60 h (f), and atretic antral follicles at 24 h (b), 42 h (c) and 60 h (e) after LHRH-AS treatment at 1100 h on day 4 of the oestrous cycle were stained with haematoxylin and eosin. Bar represents 150 μ m; all sections are shown at the same magnification.

after the LHRH-AS treatment, but the rate of ovulation and the number of ovulations gradually recovered from 36 h after the treatment.

Ovarian histology

Morphological changes in the ovary in response to LHRH-AS are shown in Figure 3. Healthy large pre-

ovulatory follicles were present in ovaries at 1100 h on day 4 (0 h). At 24 h after LHRH-AS treatment, early atretic preovulatory follicles were to be seen in the ovaries, and at 42 h after LHRH-AS treatment, new healthy antral follicles were observed, with advanced atretic preovulatory follicles in the ovary. At 60 h after LHRH-AS treatment, the number of new antral follicles was further increased, with atresia of preovulatory follicles.

Discussion

The present study clearly demonstrates that the secretory patterns of dimeric inhibin (inhibins A and B) and inhibin pro- α C are different from each other during the period of follicular atresia and the subsequent follicular development induced by LHRH-AS. In this study, atretic follicles were present in ovaries 24 h after the LHRH-AS treatment given at 1100 h on day 4 of the oestrous cycle (Fig. 3). During the first 18 h after LHRH-AS treatment, plasma concentrations of dimeric inhibins decreased, whereas plasma concentrations of inhibin pro- α C increased at 6 h after LHRH-AS treatment. Our findings in a previous study indicated that a decline in the secretion of oestradiol-17 β from antral follicles was an early sign of follicular atresia (Otsuka *et al.* 1997). Uilenbroek *et al.* (1998) have also reported that a rapid decline in the ability of preovulatory follicles to secrete oestradiol-17 β *in vitro* appeared to be an early sign of atresia. In the present study, plasma concentrations of oestradiol-17 β remained high until 6 h and declined abruptly at 12 h after the treatment, corresponding with the changes in the number of follicles in response to hCG, which suggests that follicles became atretic from 6 h to 12 h after LHRH-AS treatment. The decline in plasma inhibin pro- α C after treatment with LHRH-AS was delayed by 6–12 h as compared with that of oestradiol-17 β , suggesting that inhibin pro- α C is secreted not only from healthy follicles but also from early atretic antral follicles. Immunohistochemical localization of the inhibin α subunit in early atretic follicles observed in our previous studies (Otsuka *et al.* 1997) also supports this hypothesis.

Dimeric inhibins are secreted mainly from granulosa cells of antral follicles in the hamster ovary during the oestrous cycle (Kishi *et al.* 1995, Ohshima *et al.* 1999). In the present study, plasma concentrations of inhibin A did not recover completely from the decline during the experimental period, whereas those of inhibin B recovered by 42 h after the LHRH-AS treatment, when new antral follicles were present. Furthermore, changes in inhibin A were associated with changes in the ovulatory response of follicles to hCG throughout the experiment. In addition, a surge of inhibin A, noted at 1700 h on day 4 in the control animals, disappeared with blockade of the LH surge after LHRH-AS treatment, suggesting that the LH surge may induce a preovulatory surge of inhibin A. Greenwald (1967) demonstrated that the number of follicles larger than 415 μ m in diameter had a relationship with the number of ovulations induced by hCG throughout pregnancy in the hamster. These results indicate that inhibin A is mainly secreted from mature preovulatory follicles. This view is also supported by our previous study showing that the changes in plasma concentrations of inhibin A reflect the number of large antral follicles in the ovary (Ohshima *et al.* 1999). In the present study, plasma concentrations of inhibin B recovered earlier than those of inhibin A. Kishi

et al. (1995, 1997b) have reported that inhibin has a major role in regulating the ovulation rate in the hamster through the control of FSH secretion. The secondary FSH surge starts to decrease with increases in inhibin A and inhibin B in the normal oestrous cycle of the cyclic hamster (Ohshima *et al.* 1999). In the present study, however, inhibin B alone increased when plasma concentrations of FSH decreased. A significant negative relationship between the pattern of FSH and inhibin B in the plasma was also observed. These results suggest that inhibin B secreted from small antral follicles might play a major role in suppressing the secondary FSH surge in the cyclic hamster.

In the current study, plasma concentrations of progesterone increased abruptly at 30 h after LHRH-AS treatment, and no ovulatory response to hCG injected at this time was noted during this period. Hubbard & Greenwald (1981) demonstrated that, after atresia was induced in antral follicles of hypophysectomized pregnant mare's serum gonadotrophin (PMSG)-treated hamsters by an injection of PMSG antiserum, serum progesterone showed a sharp increase. These results suggest that atretic follicles secrete progesterone.

Conclusions

The present data suggest that inhibin A is mainly secreted by mature preovulatory follicles, whereas inhibin B is secreted by small and large antral follicles. Inhibin pro- α C is secreted from not only healthy follicles, but also from early atretic antral follicles.

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