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Changes in Inhibin Secretion during Development of the Female Duck Embryo

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ABSTRACT—Concentrations of immunoreactive (ir-) inhibin in circulation, amniotic fluid and the ovary of embryos and newly-hatched ducks from Day 21 of incubation to one day of age were determined. The antiserum used was against bovine 31-kDa inhibin and was validated for RIA of inhibin using samples from female embryos. Plasma concentrations of FSH and LH were also determined by chicken RIA systems. Plasma concentrations of ir-inhibin were maintained at constant levels from day 21 to day 25, abruptly increased on day 26. After the increase, plasma concentrations of ir-inhibin quickly declined from day 27 to the day of hatch (day 28). Plasma concentrations of FSH were high on day 21, followed by an abrupt decline on day 22, gradually increased until day 24, and then decreased again on day 25. The maximal rise of plasma FSH was observed on day 27, and then rapidly decreased until the day after the hatch. No inverse relationship was observed between plasma concnetrations of ir-inhibin and FSH. Amniotic fluid concentrations of ir-inhibin were relatively low and remained constant between Day 21 and Day 25. Embryonic ovaries contained very low amounts of ir-inhibin. Ovarian inhibin levels were stabilized in early time points, temporally decreased on day 25, and abruptly increased on day 26. These results suggest that inhibin does not involved in the regulation of FSH secretion in the female duck embryo and the ovary of duck embryo is not the main source of circulating ir-inhibin.

Key words: duck, embryo, inhibin, FSH, LH

INTRODUCTION

Inhibin is a glycoprotein hormone composed of α-subunit and one of the two β-subunits, βA or βB (Burger *et al*., 1988). Homodimers of the β-subunits forms activins (Vale *et al*., 1988). In mammalian females, inhibin is mainly secreted from ovarian granulosa cells (Steinberger *et al*., 1976). Inhibins specifically inhibit FSH secretion (Vale *et al*., 1988), whereas activins have the opposite effect (Schwall *et al*., 1989). In addition, inhibins and activins have been shown to exert paracrine/autocrine effects on steroidogenesis and folliculogenesis in the ovary (Findlay, 1993). Chick inhibin is likely to play important roles in the regulation of pituitary FSH secretion (Akashiba *et al*., 1988; Tsonis *et al*., 1988; Vanmontfort *et al*., 1992; Johnson *et al*., 1993a, b) as well as mammalian inhibin. In the hen, the ovarian follicles have been demonstrated to be a major source of circulating immunoreactive (ir-) inhibin (Vanmontfort *et al*., 1992; Johnson *et al*., 1993a, b).

Rombauts *et al*. (1992) have reported that high concentrations of plasma ir-inhibin are observed during the chick embryonic development. The following studies accomplished by the same group indicated that the main source of circulating inhibin in the chick embryo is the embryonic adrenal gland rather than the gonad (Rombauts *et al*., 1993, 1994). Relatively a low amount of ir-inhibin was present in chick embryonic ovary as compared to other tissues studied, whereas appreciable concentrations of ir-inhibin was observed in circulation of both sex embryos (Rombauts *et al*., 1992, 1993). The origin and roles of inhibin in female avian embryos are unique and of interest.

Accumulating numbers of evidence support the existence

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of a functional FSH-inhibin feedback system in male ovine fetus (Albers *et al.*, 1989a, b). In contrast, the FSH-inhibin feedback system does not exist in chick embryos (Rombauts *et al.*, 1993). Furthermore, the inhibin secretion of female chick embryos is likely to be independent from the pituitary regulation (Rombauts *et al.*, 1993). *In vitro* study has suggested the paracrine effect of inhibin and activin on steroidogensis of gonadal cells (Rombauts *et al.*, 1996). It is likely that ovarian inhibin plays an intraovarian factor as well as an FSH suppressing endocrine factor.

Despite these previous investigations, information about the exact nature and physiological roles of this material in the avian species is still relatively limited and most of the studies of avian inhibin have been carried out using the chicken. In the present study, we used the duck as an experimental animal in order to extend the knowledge of inhibin in avian species. Furthermore, it is important to elucidate the reproductive physiology of the duck because the duck is one of the most important domestic avian species in the world as well as the chicken. In the present study, changes in plasma concentrations of ir-inhibin in the duck embryo were investigated. In addition, changes in ovarian contents and amniotic concentrations of ir-inhibin and plasma concentrations of two pituitary gonadotropins were examined in duck embryos during the late part of development.

MATERIALS AND METHODS

Animals and tissues

Fertilized eggs of British Khaki Campbell duck were incubated at 37.5±0.2°C and 70% humidity. Amniotic fluid, blood and the ovaries were collected from embryos of 21 days of incubation through one-day old hatching. Amniotic fluid was aspirated through the intact amniotic membrane. After 25 days of incubation too little fluid remained to allow adequate collection. Embryos were put on ice, dissected and sexed. Day-old hatching ducks were anesthetized with ether before autopsy. Blood samples were collected from the heart into heparinized tubes. After centrifugation for 10 min at 1,700 g, 4°C, plasma and amniotic fluid were stored at –20°C until assay. Ovaries were dissected from female embryos, weighted and stored in 100 μl saline at –20°C until homogenization. Ovarian samples were homogenized in 500 μl saline. All the homogenates were centrifuged at 20,000 g for 30 min at 4°C. The supernatants were collected and stored at –20°C until assay.

RIAs for linhibin, FSH, and LH

Concentrations of plasma inhibin were measured using a rabbit antiserum against bovine inhibin (TNDH-1) and ^{125}I-labeled 32-kDa bovine inhibin as described previously (Hamada *et al.*, 1989). The inhibin RIA showed no significant cross reaction with LH, FSH and prolactin of rats, cattle and sheep, and with GnRH, transforming growth factor, and activin, whereas the assay system cross-reacts with inhibin Pro-αC and free inhibin α-subunit (Kaneko *et al.*, 1995). Results were expressed in terms of 32-kDa bovine inhibin. The intra- and inter-assay coefficients of variation were 6.2 and 7.4%, respectively.

Highly purified chicken FSH (AGCQSQ 111232D) and anti-chicken FSH serum raised in a rabbit were kindly supplied by Dr. S. Ishii. The FSH RIA was done as described by Sakai and Ishii (1980) with modifications. Iodination was accomplished by chloramine-T procedure. The standard used was chicken gonadotrophin standard fraction (AGC112B). The relative potencies of this standard to the highly purified chicken FSH and LH are 0.0075 and 0.13, respectively (Kikuchi *et al.*, 1989). Results were expressed in term of chicken FSH (AGCQSQ 111232D). The intra- and inter-assay coefficients of variation were 6.0 and 7.4%, respectively.

Plasma concentrations of LH were determined by a double-antibody RIA system using ^{125}I-labeled radioligands as described previously (Mauget *et al.*, 1994). The iodinated preparation and antiserum used were purified chicken LH (AGMS1122F, provided by Dr. S. Ishii, Waseda University, Japan), and anti-chicken LH (HAC-CH27-01RBP75, kindly provided by Dr. K. Wakabayashi, Gunma University, Japan). Results were expressed as purified chicken LH (AGMS1122F). The intra- and inter-assay coefficients of variation were 5.4 and 8.2%, respectively.

Two-site enzyme-linked immunosorbent assay (ELISA) for inhibin A and inhibin B

Inhibin A and inhibin B were measured using the ELISA kits (Serotec, Oxford, UK) (Muttukrishna *et al.*, 1994; Groome *et al.*, 1996) for the measurement of human inhibin A and inhibin B without modifications. Serial dilution of plasma and testicular homogenate of embryos were assayed to test parallelism.

Statistics

Values are expressed as means±S.E.M. The statistical significance of the difference between means was assessed by one-way analysis of variance, followed by the Student's t-test when the variance was uniform. When the variance was not uniform, the Cochran-Cox test was performed. Values of $P<0.05$ were considered to be statistically significant.

RESULTS

Validation of the Inhibin RIA System (Fig. 1)

About 25% of radioiodinated 32-kDa bovine inhibin bound to antiserum at a final dilution of 1:100 000. Displacement of

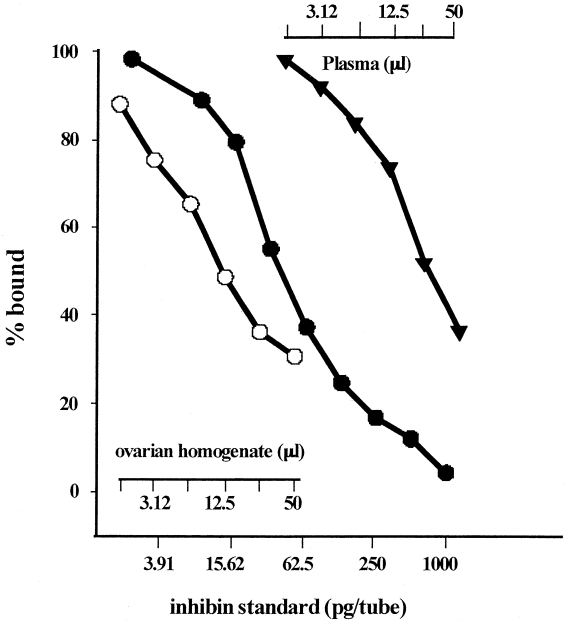

Fig. 1. Dose-response curves of bovine 32 kDa inhibin (closed circles), embryonic ovarian homogenates (open circles) and diluted peripheral pooled plasma (closed triangles) from female duck embryos in the inhibin radioimmunoassay. Each value represents the mean of triplicate determinations.

tracer with partially purified bovine inhibin, duck embryonic ovarian homogenates and peripheral pooled plasma from duck female embryos produced suitable dose-response curves. These curves were parallel with the bovine inhibin standard curve, indicating that it was possible to measure the concentrations of inhibin in the duck using this bovine inhibin RIA system.

Measurement of Inhibin and FSH in plasma from Day 21 of Incubation to 1 Day of Age (Fig. 2)

From Day 21 to Day 25 of incubation, plasma ir-inhibin maintained at low levels. Thereafter, plasma ir-inhibin significantly increased (P<0.05) and reached the maximum level on Day 26 and remained high until Day 27. Afterwards, plasma ir-inhibin abruptly decreased on the day of hatch (P<0.05). Plasma FSH significantly decreased from Day 21 to Day 22, gradually elevated until Day 24, and decreased on Day 25. From Day 25 to Day 27, plasma FSH gradually increased to the maximum level on Day 27, and then noticeably decreased until one day of age.

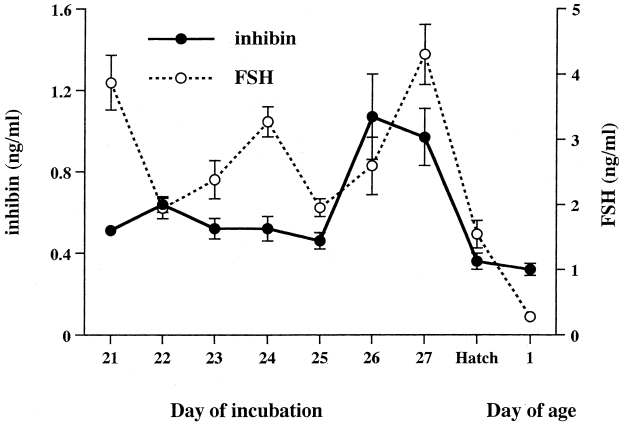

Fig. 2. Changes of plasma immunoreactive inhibin (closed circles, solid lines) and FSH (open circles, dotted lines) concentrations in duck embryos and newly-hatched ducks from Day 21 of incubation to 1 day of age. Results are the mean±SEM of 5 animals.

Ovarian Inhibin Concentrations from Day 21 of Incubation to 1 Day of Age And Amniotic Fluid Inhibin Concentrations (Fig. 3)

Ovarian inhibin contents were unchanged from Day 21 to Day 24, decreased and reached the nadir on Day 25, and then abruptly increased on Day 26. Thereafter, ovarian inhibin decreased a little and remained constant levels from Day 27 onward. Amniotic fluid concentrations remained at constant levels, and were lower than those in fetal plasma from Day 21 to Day 25.

Plasma LH Concentrations from Day 21 of Incubation to 1 Day of Age (Fig. 4)

A maximum level of plasma LH was observed on Day 21. Plasma LH continuously decreased until Day 25, and recovered by Day 27. Thereafter, plasma concentrations of LH slightly decreased on the day of hatch.

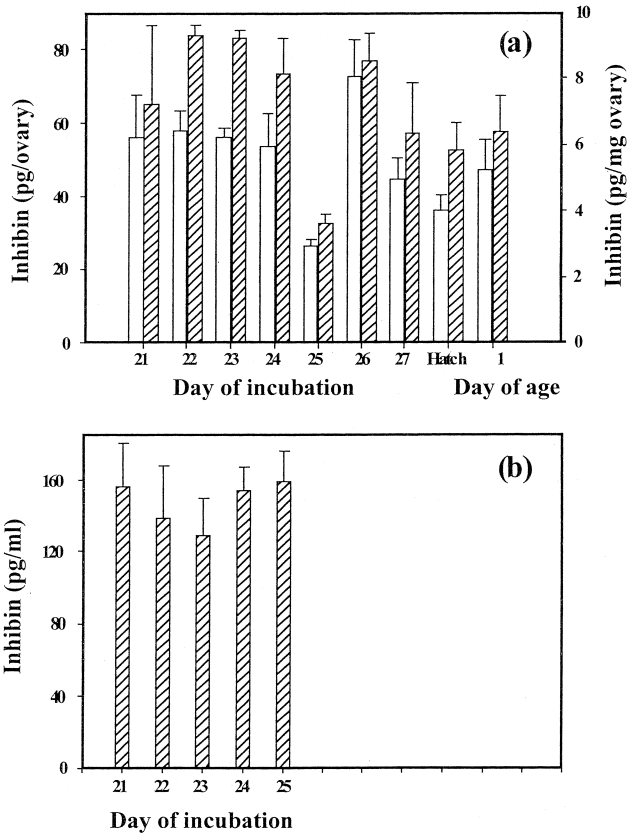

Fig. 3. Immunoreactive inhibin contents in ovaries from Day 21 of incubation to 1 day of age (**a**) and inhibin concentrations in amnoitic fluid from Day 21 to Day 25 of incubation (**b**). Results are expressed per ovary (**a**, hatched bars) and per milligram of wet weight (**a**, open bars), respectively. Results are the mean±SEM of 5 animals.

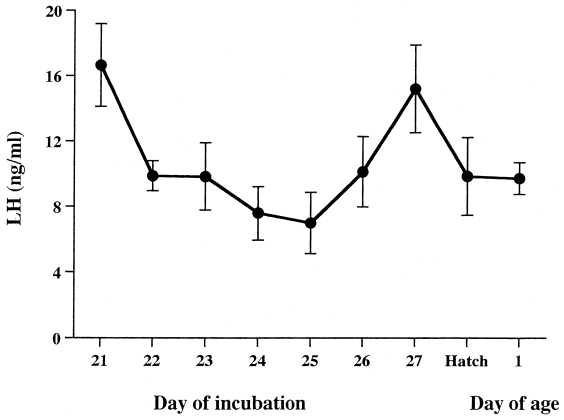

Fig. 4. Changes of plasma LH concentrations in duck embryos and newly-hatched ducks from Day 21 of incubation to 1 day of age. Results are the mean±SEM of 5 animals.

Characterization of the dimeric inhibin ELISA system (Fig. 5)

Serial dilution of pooled female duck embryo plasma gave dose-response curves in both inhibin A and inhibin B ELISAs

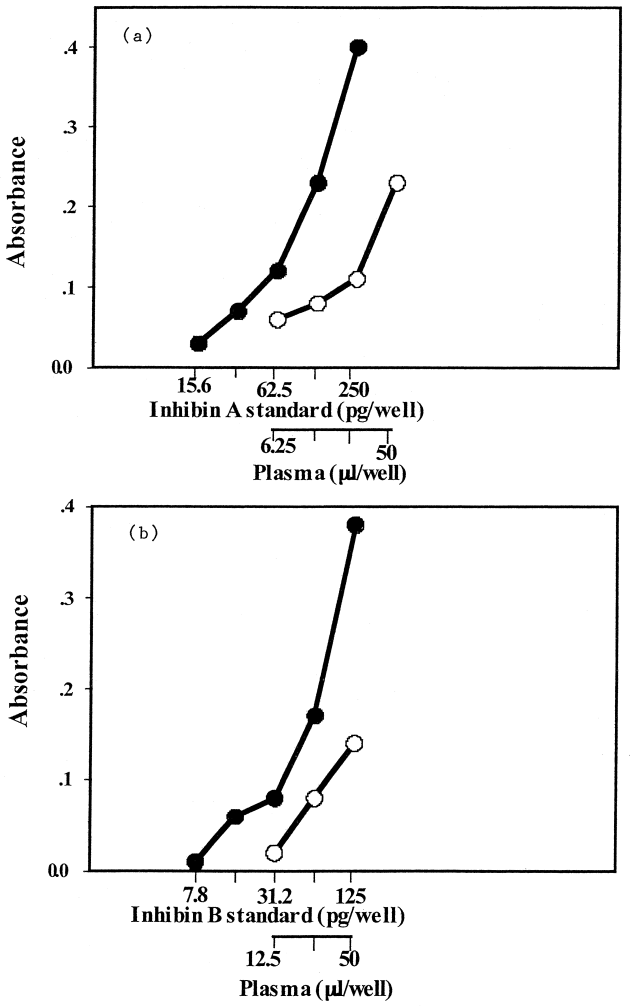

Fig. 5. Dose-response curves in the two-site ELISA for inhibin A (**a**) and inhibin B (**b**), showing parallelism between the respective standard (closed circles) and serial dilutions of pooled plasma from duck embryos (open circles). Values are the means of duplicate determinations and experiments repeated in twice.

that were parallel to the respective standard curve (Fig. 5a and b). These results indicate that the assay systems were applicable for measuring plasma concentrations of inhibin A and inhibin B in duck female embryos.

DISCUSSION

In the present study, the characteristics of inhibin secretion in female duck embryos were examined with particular reference to the relationship between plasma ir-inhibin and gonadotropins. The contribution of embryonic ovary to the circulating ir-inhibin was estimated by comparing ovarian inhibin content with plasma inhibin concentrations.

Plasma ir-inhibin concentrations showed significant changes during the period studied. An increase with a peak on Day 26 was observed, and was followed by a marked drop towards hatch. The pattern of plasma ir-inhibin was similar to that reported in chick female embryos with the difference of peak-value time. The latter achieved peak-value at Day 13. This difference may be due to the total incubation time. Duck embryos have longer developmental period (28 days) compared to that of chicken (20 days).

There is no inverse or positive relationship between ir-inhibin and FSH in developing female duck embryos in the present study. This is consistent with the observation in female chick embryos (Rombauts *et al.*, 1993). The patterns of plasma ir-inhibin and FSH in the present study suggest that most of the measured ir-inhibin is not involved in the control of FSH secretion in the duck. However, the present results showed that dimeric inhibins are present in the embryonic plasma. This is the first demonstration of the existence of dimeric inhibin in plasma of the avian embryo. The results strongly indicate that the ovary of the duck embryo secretes biologically active inhibins.

Plasma concentrations of FSH in the chick female embryo are relatively low compared with those observed in the male chick embryo (Rombauts *et al.*, 1993). This may be due to higher biological activity of ir-inhibin produced by the embryonic ovary than that produced by embryonic testis as already demonstrated in the bovine fetus (Torney *et al.*, 1992). The present results of the inhibin ELISAs also indicate that a large amount of biologically active inhibins are present in the circulation of female duck embryos. On the other hand, Rombauts *et al.* (1993) suggest that high concentrations of estradiol produced by the embryonic chicken ovary may be responsible for the suppression of pituitary FSH secretion in the female embryo. Although plasma concentrations of estradiol in the female duck embryo were not examined in this study, estradiol may be involved in the suppression of FSH secretion in the female duck embryo as well as the chicken embryo.

High concentrations of ir-inhibin were measured in circulation but low levels of ir-inhibin were detected in embryonic ovarian homogenates. In agreement with the present results, high concentrations of ir-inhibin were found in plasma but low levels of ir-inhibn were observed in embryonic ovaries in the chicken (Rombauts *et al.*, 1992). Circulating ir-inhibin concentrations were about 10-fold of those in the ovary. Furthermore, circulating ir-inhibin and ovarian contents of ir-inhibin showed different patterns during female duck development. These results suggest that other tissue sources of inhibin apart from the gonad contribute to the plasma ir-inhibin. Rombauts *et al.* (1992) have reported the same results in chicken embryos and concluded that the gonads may not be the most significant source of plasma ir-inhibin in the chick embryo. The adrenal gland has been postulated to be a main source of circulating inhibin in the chicken embryo (Rombauts *et al.*, 1994). With respect to mammals, the fetal gonad is likely to be a major source of inhibin in male but not in female ovine fetal plasma (Miller *et al.*, 1997). In the male ovine fetus, gonadectomy considerably decrease fetal circulating inhibin. However, in the report, fetal plasma inhibin was measurable even after the gonadectomy and the levels of plasma inhibin were not different from that in female fetus. Furthermore, the adrenal gland is likely to be a main source of circulating

ir-inhibin in the baboon fetus (Billiar *et al*., 1999). Therefore, in mammals, adrenal gland is probably an important source of ir-inhibin as well as avian species.

Immunoreactive inhibin was also detected in amniotic fluid. These levels remained relatively constant and did not reflect the changes in plasma ir-inhibin concentrations as reported in the chick embryo (Rambauts *et al*., 1992), suggesting that the source of inhibin in the amniotic fluid is different from that in embryonic plasma.

The pattern of plasma LH during the development of duck embryo is consistent with the previous observations in chick embryo (Tanabe *et al*., 1986). In chick embryonic testicular cell culture, LH has the stimulative effect on inhibin secretion as well as FSH (Rombauts *et al*., 1995). Therefore, high concentrations of plasma LH at early points of sampling can be speculated to have the stimulative effect on ovarian inhibin secretion. The decrese in plasma LH until day 25 of incubation may be responsible for the decrease in ovarian contents of inhibin on day 25. Furthermore, the increase in plasma LH during day 26 and 27 of incubation may be responsible for the recover of ovarian contents of inhibin.

In conclusion, present results suggest that inhibin does not involved in the regulation of FSH secretion in the female duck embryo and the ovary of duck embryo is not the main source of circulating ir-inhibin.

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