Introduction

In mammals, inhibins and activins have been implicated in the negative and positive feedback regulation of FSH, respectively (for reviews see Woodruff and Mather, 1995; Knight, 1996). Although it has been established that inhibin A and activin A dimers are synthesized in preovulatory follicles of the domestic hen (Lovell et al., 1998), their presence in the peripheral circulation has not yet been investigated. A potential endocrine role of inhibin is indicated by the fact that the decrease in circulating immunoreactive inhibin α-subunit (ir-α-inhibin) concentrations after selective removal of ovarian follicles is accompanied by an increase in plasma FSH concentrations (Johnson et al., 1993a). However, neither Johnson et al. (1993b) nor Vanmontfort et al. (1994) found any significant variation in plasma ir-inhibin during the ovulatory cycle of the hen. It is necessary to interpret data on ‘first generation’ inhibin radioimmunoassays with caution, as it is now generally accepted that these assays crossreact freely with inhibin α-subunit forms that are known to be secreted in large amounts by the mammalian ovary (Knight et al., 1989; Schneyer et al., 1990; Robertson et al., 1995). Chen and Johnson (1997) measured the steady-state content of mRNA encoding the α- and β₁- subunits of inhibin at 4 h intervals throughout the ovulation cycle. The β₁-subunit mRNA content was low in F₂ follicles and in F₁ follicles at 23.5 h before ovulation; it increased to its highest value between 19.5 and 11.5 h before

Circulating concentrations of inhibin-related proteins during the ovulatory cycle of the domestic fowl (Gallus domesticus) and after induced cessation of egg laying

T. M. Lovell¹, D. Vanmontfort², V. Bruggeman², E. Decuypere², N. P. Groome³, P. G. Knight¹ and R. T. Gladwell¹*

¹School of Animal and Microbial Sciences, The University of Reading, Whiteknights, Reading RG6 6AJ, UK; ²Department of Animal Science, Catholic University of Leuven, Leuven, Belgium; and ³School of Biological and Molecular Sciences, Oxford Brookes University, Oxford OX3 0BP, UK

Circulating inhibin A, inhibin B, activin A, total immunoreactive inhibin α-subunit (ir-α-inhibin), LH, FSH and progesterone concentrations were measured throughout the normal ovulatory cycle and after cessation of egg laying induced by feed restriction to investigate the potential involvement of inhibins and activins in the ovulatory cycle of the domestic hen. Plasma inhibin A varied significantly (P < 0.05) during the ovulatory cycle; the concentration was highest at the preovulatory LH surge and reached a nadir 10 h later, at about the time the F₂ follicle makes the transition to become the new F₁ follicle. Plasma FSH concentrations did not change significantly throughout the cycle and showed no correlation with inhibin A. Total ir-α-inhibin concentrations were much higher than those of inhibin A at all stages of the ovulatory cycle and showed no correlation with inhibin A or FSH. Plasma concentrations of inhibin B and of activin A were below the detection limit of the assays in all plasma samples analysed. In the feed restriction study, plasma inhibin A and total ir-α-inhibin showed little change until the last day of oviposition (day 0) after which they fell significantly (P < 0.05) and remained low to the end of the experiment (approximately 70–78% decrease relative to day –4). Conversely, plasma FSH increased after cessation of laying and was significantly higher (P < 0.05) from day 3 to the end of the study (approximately 50% increase on day 6 relative to day –4). Plasma FSH values were negatively correlated with inhibin A (r = –0.39; P < 0.005) and total ir-α-inhibin (r = –0.36; P < 0.005). Plasma LH and progesterone also decreased (P < 0.05) during feed restriction. The decrease in LH preceded the terminal oviposition and the associated fall in inhibin A by 2 days; there was a positive correlation between LH and inhibin A (r = 0.35; P < 0.005). Taken together these findings support (i) a role for LH in promoting inhibin A secretion by preovulatory follicles and (ii) an endocrine role for inhibin A secreted by preovulatory follicles in the maintenance of tonic FSH secretion in laying hens.

*Correspondence.
Revised manuscript received 17 March 2000.
ovulation, and thereafter decreased until ovulation. Inhibin α-subunit mRNA was abundant and did not vary throughout the ovulatory cycle in either the F₁ or F₂ follicles. These changes in subunit mRNA content are supported by the observation that the inhibin A (α-β₂, dimer) content is highest in the F₁ follicle and at a maximum at 12 h before ovulation (Lovell et al., 1998).

The objective of the present study was to measure plasma inhibin A, inhibin B and activin A concentrations during the ovulatory cycle of the hen and after induced cessation of egg laying, a procedure that leads to ovarian atresia (Decuyper et al., 1992), to gain an insight into potential endocrine roles of inhibin and related dimers in the avian reproductive system. Plasma concentrations of LH, FSH, progesterone and ir-α inhibin were reported in Vanmontfort et al. (1994). In this study the stored plasma samples were assayed for inhibin A, inhibin B and activin A using specific two-site ELISAs (Muttukrishna et al., 1994; Groome et al., 1996; Knight et al., 1996) that were validated for use with chicken plasma.

**Materials and Methods**

**Experimental animals**

Laying hens (ISA Brown), 40–50 weeks of age, with a clutch average of at least five eggs were caged individually and maintained under a standard long-day photoperiod (16 h light: 8 h dark) at an ambient temperature of 21–23°C with food and water freely available. Ovipositions were recorded each day.

**Experimental design**

In Expt 1, serial blood samples were taken from laying hens (n = 10) at 2 h intervals for 36 h via a cannula inserted into the brachial vein. The cannula was inserted on the day before the initiation of blood sampling. Blood samples were collected into heparinized tubes, centrifuged at 3000 g for 20 min at 4°C and the plasma was stored at −20°C before analysis. In Expt 2, ten laying hens were subjected to an induced moulting programme (ADAS method: 1 day of food withdrawal followed by 20 days of restricted wheat bran, according to Wakeling (1985)). Blood samples (2 ml) were taken from a brachial vein by venepuncture into heparinized tubes during ten consecutive days; the first blood sample was taken immediately before food withdrawal. Blood samples were centrifuged at 3000 g for 20 min at 4°C and the plasma was stored at −20°C until required. At the end of Expt 2, the degree of ovarian atrophy was assessed visually. Samples from Expts 1 and 2 were assayed for total ir-α inhibin, LH, FSH and progesterone and the results were reported in Vanmontfort et al. (1994). The same samples were assayed for activin A, inhibin A and inhibin B.

**Immunooassays**

Inhibin A, inhibin B and activin A were determined using two-site ELISAs that involve monoclonal antibodies raised against synthetic peptide fragments of the human α-, β₂- and β₅-subunits (Muttukrishna et al., 1994; Groome et al., 1996; Knight et al., 1996). Assays were validated for use with hen plasma as described below, although it should be noted that the inhibin A and activin A ELISAs have been validated for hen ovarian tissue extracts (Lovell et al., 1998). Total ir-α inhibin concentrations were measured using a heterologous radioimmunoassay involving a rabbit polyclonal antiserum against purified bovine inhibin (Robertson et al., 1988) that has been validated previously for use in domestic fowl (Rombaerts et al., 1992). The ir-α inhibin values are expressed relative to a recombinant human inhibin A standard rather than an internal standard as reported by Vanmontfort et al. (1994). Recombinant human (rh) activin A, rh-inhibin B and rh-inhibin A were used as assay standards. Plasma samples were also assayed for LH, FSH and progesterone by radioimmunoassays as described by Vanmontfort et al. (1994, 1995).

**Statistical analysis**

Confirmation of parallelism between assay standards and test sample dilution curves was made using linear regression analysis of transformed data. The log-log transformation was used to linearize the ELISA dose–response curves. Comparison of the slopes (±95% confidence intervals) of the regression lines for standards and test samples indicated no significant departure from parallelism. One-way repeated measures ANOVA was used in conjunction with post hoc Fisher’s protected least significant difference (PLSD) test to determine whether concentrations of inhibin A, ir-α inhibin, LH, FSH and progesterone varied at the different time points. Post hoc tests were only performed when the ANOVA yielded a significant F ratio. P<0.05 was considered to be significant. Unless otherwise stated, values are the means ± SEM. Ovulatory cycle data from Expt 1 are expressed relative to the time of the LH surge, whereas data from Expt 2, involving hens subjected to induced ovarian regression, are expressed relative to the day of last oviposition.

**Results**

**Validation of inhibin A, inhibin B and activin A assays for chicken plasma samples**

Serial dilutions of pooled plasma samples (juvenile and adult) gave response curves in the ELISA for inhibin A that were parallel to the rh-inhibin standard (see Fig. 1) and mean recovery of exogenous inhibin A added to hen plasma before assay was quantitative (103%). Plasma concentrations of inhibin B and activin A were below the detection limits of the respective assays. Despite this recovery of exogenous inhibin B and activin A standard added to hen plasma was apparently quantitative (means: inhibin B, 91%; activin A, 96%) indicating that endogenous concentrations were very low. The minimum detection limits of the inhibin A, inhibin B and activin A ELISAs were 2, 12 and 50 pg ml⁻¹, respectively. The low concentrations of inhibin B and activin A were not
artefacts due to storage of the samples as in plasma samples from 11 laying hens stored for less than 1 week before assay their concentrations were below the minimum detectable limit; moreover, inhibin A concentrations were very similar in both sets of samples.

The crossreactivities of a range of substances in the inhibin A, inhibin B and activin A ELISAs have been reported (Muttukrishna et al., 1994; Groome et al., 1996; Knight et al., 1996) and were acceptably low. Intra- and inter-plate coefficients of variation were < 10% in each case.

Inhibin A, ir-α inhibin, progesterone, LH and FSH during the hen ovulatory cycle

Plasma concentrations of inhibin A, ir-α inhibin, progesterone, LH and FSH during the hen ovulatory cycle are shown (Fig. 2). The expected preovulatory peaks for LH and progesterone were evident. Inhibin A showed a small yet significant ($P < 0.05$) pattern of change throughout the ovulatory cycle. Concentrations peaked at the time of the LH surge and reached a nadir 10 h later (approximate twofold reduction). Plasma concentrations of ir-α inhibin did not vary significantly during the ovulatory cycle ($P < 0.05$) and were higher than those of inhibin A at all times. There was no relationship between the concentrations of ir-α inhibin and inhibin A. FSH concentrations did not vary significantly throughout the cycle and thus were not correlated with plasma inhibin A or ir-α inhibin concentrations. As mentioned above, activin A and inhibin B concentrations were below the detection limit of the assay throughout the ovulatory cycle.

Inhibin A, ir-α inhibin, progesterone, LH and FSH during induced cessation of egg laying

Plasma concentrations of inhibin A, ir-α inhibin, progesterone, LH and FSH during induced cessation of laying are shown (Fig. 3); data are expressed relative to the last day of oviposition. Inhibin A concentrations did not vary significantly until after the last day of oviposition (day 0). From day 1 they were significantly ($P < 0.05$) lower than those at day –4, reaching a nadir on day 4 (approximate 70% decrease relative to day –4). Total ir-α inhibin concentrations were relatively uniform until day –1; concentrations subsequently decreased relative to day –4, reaching significance ($P < 0.05$) by day 1 and reaching minimum values (approximate 77% decrease relative to day –4) from day 4 to the end of the experiment. Plasma ir-α inhibin and inhibin A concentrations were positively correlated throughout this study ($r = 0.57; P < 0.001$). Plasma
Inhibin A and LH concentrations during induced ovarian regression were positively correlated ($r = 0.346$; $P < 0.003$). Plasma progesterone concentrations were also significantly reduced ($P < 0.05$) during induced ovarian regression; values were significantly lower on day 0 and days 2–6 and reached a nadir on day 6 (approximate 60% decrease relative to day 4). Circulating concentrations of progesterone and FSH were negatively correlated during induced cessation of laying ($r = -0.27$; $P < 0.005$).

**Discussion**

Although changes in circulating concentrations of reproductive hormones throughout the ovulatory cycle of laying hens are well documented (Etches, 1990) this study represents the first attempt to measure plasma dimeric inhibins (A and B) and activin A in an avian species. The main findings with respect to the normal ovulatory cycle were: (i) inhibin A concentrations changed in a defined pattern throughout the ovulatory cycle, increasing to a peak at the time of the LH surge, and decreasing to a nadir 10 h later; and (ii) plasma FSH and ir-$\alpha$ inhibin concentrations did not change significantly throughout the ovulatory cycle, and showed no correlation with inhibin A concentrations. Preovulatory peaks of LH and progesterone were observed, which are consistent with other studies (Furr, 1973; Wilson and Sharp, 1973; Etches and Cunningham, 1976; Johnson and van Tienhoven, 1980).

The absence of significant changes in plasma concentrations of total ir-$\alpha$ inhibin during the ovulatory cycle confirm the findings of Johnson et al. (1993b), although in that study the highest concentrations were attained 2 h before the preovulatory LH surge. This is a similar time to the inhibin A peak detected in the present study by the two-site ELISA. The high plasma concentrations of ir-$\alpha$ inhibin relative to those of dimeric inhibin A reflect its 10–100-fold excess over dimeric inhibin A contents in hen preovulatory follicle granulosa and theca layers (Lovell et al., 1998). This excess is also apparent in the mRNA content of preovulatory follicle granulosa tissue, which contains between 70- ($F_3$ follicle) and sevenfold ($F_1$ follicle) more mRNA for inhibin $\alpha$-subunit than for inhibin–activin $\beta_a$-subunit (Chen and Johnson, 1996a). The cellular content of inhibin–activin $\beta_a$-subunit may therefore be a key determinant in the rate of inhibin A synthesis. Clearly, results obtained by the use of the conventional ‘Monash’ radioimmunoassay grossly overestimate the effective inhibin dimer concentration in samples and mask cyclic changes related to specific events of the ovulatory cycle.

Domestic fowl have an ovulatory cycle of approximately 26 h (Etches et al., 1990) and the preovulatory LH peak occurs about 6 h before ovulation. The mRNA encoding the $\beta_a$-subunit of inhibin is expressed in all the hierarchical follicles, but predominantly in the $F_1$ follicle (Davis and Johnson, 1998; Safi et al., 1998). The time of the lowest plasma inhibin A concentration (22 h before ovulation) corresponds to the presence of a newly promoted $F_1$ follicle at about the time (23.5 h before ovulation) that $F_1$ granulosa cell $\beta_A$-subunit mRNA expression is at its lowest (Chen and Johnson, 1997).

**Fig. 3.** Plasma concentrations of inhibin A (●), FSH (○), LH (■), progesterone (□) and total immunoreactive inhibin α-subunit (∇) in plasma from hens in which cessation of egg laying was induced by food restriction. All data are plotted relative to the day of last oviposition (day 0) and values are means ± SEM ($n = 5–8$). Asterisks denote significant differences ($P < 0.05$) compared with day –4. Data for FSH, LH, progesterone and total immunoreactive α-subunit were reported in Vanmontfort et al. (1994).
Maximum expression of inhibin–activin $\beta_A$-subunit mRNA in granulosa cells from F$_1$ follicles was about 5 h before the peak of plasma inhibin A. There was a significant reduction in plasma inhibin A dimer (this study) and F$_1$ granulosa cell $\beta_A$-subunit mRNA expression (Chen and Johnson, 1997) after the LH surge, indicating that the production and secretion of inhibin A from the F$_1$ follicle may be attenuated after the LH surge. Further support for this contention arises from the observations that administration of exogenous LH to hens reduces inhibin–activin $\beta_A$-subunit mRNA expression in F$_1$ folliculosa cells without altering $\alpha$-subunit mRNA expression (Chen and Johnson, 1996b). Furthermore, the inhibin A protein content of F$_1$ granulosa cells was sixfold lower at 4 h before ovulation (after the preovulatory LH surge) than at 12 h before ovulation (Lovell et al., 1998).

Neither inhibin B nor activin A were detectable in hen plasma either during the ovulatory cycle or in food restricted hens undergoing ovarian atrophy. Although activin A concentrations were below the detection limit of the assay, an endocrine role cannot be discounted since activin A is produced in significant amounts in the theca layers of preovulatory follicles (Lovell et al., 1998). The effect of induced ovarian regression on circulating hormones was investigated by subjecting hens to a programme of food restriction, which is known to disrupt follicle development and induce cessation of laying. The involution of the ovary may be initiated by the hypothalamic–pituitary axis as the pituitary gland becomes entirely refractory to stimulation by exogenous GnRH (Etches, 1990). Cessation of egg laying was accompanied by a progressive decrease in plasma inhibin A and total $\alpha$-inhibin concentration, which fell by approximately 70 and 77%, respectively. This finding indicates that the developing follicles of the preovulatory hierarchy are the major source of circulating inhibin A and $\alpha$-inhibin in laying hens. The persistence of detectable amounts of inhibin A and $\alpha$-inhibin in plasma of food restricted hens indicates that they are also secreted by non-hierarchical follicles, or by other extragranulosa tissues. mRNA encoding the $\alpha$ and $\beta_A$-subunits of inhibin have been detected in a variety of non-ovarian tissues (Chen and Johnson, 1996c). A contribution from non-ovarian inhibin follicles is indicated by the observations of Johnson et al. (1993a) who found that the removal of the small external yellow follicles (>5 mm in diameter) reduced plasma total $\alpha$-inhibin concentrations below those obtained by removing F$_1$-r. Since expression of the inhibin $\alpha$-subunit is reported to be high in non-hierarchical (5–12 mm) follicles, but the content of inhibin–activin $\beta_A$-subunit is low (Davis and Johnson, 1988; Safi et al., 1998), the decrease in plasma $\alpha$-inhibin demonstrated by Johnson et al. (1993a) probably represents inhibin $\alpha$-subunit and not inhibin A dimer. Whether inhibin A derived from preovulatory follicles that have undergone or are still undergoing induced atresia after feed restriction contribute to plasma inhibin A concentrations requires further evaluation. The decrease in plasma inhibin A and $\alpha$-inhibin $\beta_A$-subunit concentrations after feed restriction was preceded by a decrease in plasma LH and progesterone concentrations. Decuypere et al. (1992) reported that the decrease in progesterone occurred during the second or third day after food withdrawal, and atresia occurred several days later.

An important finding of the feed restriction study was the increase in plasma FSH concentration after terminal oviposition. This result is in agreement with the findings of Johnson et al. (1993a) who demonstrated that sequential removal of preovulatory follicles was accompanied by a progressive increase in FSH. The increased FSH concentration was inversely related to inhibin A, indicating that inhibin A from preovulatory follicles may play a key role in the negative feedback regulation of FSH secretion in laying hens. Since progesterone has a stimulatory effect on FSH secretion from hen pituitary cells in vitro (Kawashima et al., 1982), the reduced plasma progesterone concentration during ovarian regression is unlikely to contribute to the increase in plasma FSH.

In conclusion, taken together with observations on mRNA content (Davis and Johnson, 1998; Safi et al., 1998) and protein content (Lovell et al., 1998), it is likely that preovulatory follicles, especially the F$_1$ granulosa layer, are the primary source of plasma inhibin A in hens. Despite the absence of a demonstrable negative relationship between FSH and inhibin A during the ovulatory cycle, the finding of an inverse correlation during induced ovarian regression is consistent with a negative endocrine feedback role for inhibin A in setting tonic plasma FSH concentrations. Tonic FSH secretion is presumably necessary for the continual recruitment of follicles into the pre-hierarchical pool at a uniform rate. The finding that plasma inhibin A concentrations are maximum at about the time of the preovulatory LH surge and that the decrease in plasma inhibin A in feed-restricted hens is preceded by a decrease in LH support a positive role for pituitary LH in maintaining inhibin secretion by preovulatory follicles.

The authors are grateful to J. Proudman (USDA, Beltsville, MD, USA) and F. J. Cunningham (University of Reading) for providing reagents for the chicken gonadotrophin assays. This work was supported by BBSRC.

**References**

Chen C-C and Johnson PA (1996a) Expression of inhibin $\alpha$ and inhibin/activin $\beta_A$-subunits in the granulosa layer of the large preovulatory follicles of the hen. *Biology of Reproduction* 55:450–454

Chen C-C and Johnson PA (1996b) LH decreases mRNA for inhibin/activin $\beta_A$-subunit in the largest preovulatory follicle of the hen. *Biology of Reproduction (Supplement)* 52:157 (Abstract 403)


Chen C-C and Johnson PA (1997) Expression and regulation of mRNA for inhibin/activin $\alpha$- and $\beta_A$-subunits in the granulosa layer of the two largest preovulatory follicles during the hen ovulatory cycle. *General and Comparative Endocrinology* 107:386–393


Etches RJ and Cunningham FJ (1976) The interrelationship between progesterone and luteinizing hormone during the ovulation cycle of the hen (Gallus domesticus). *Journal of Endocrinology* 71:51–58

Downloaded from Bioscientifica.com at 05/10/2022 01:32:49PM via free access
Furr BJA (1973) Radioimmunoassay of progesterone in peripheral plasma of the domestic fowl in various physiological states and in follicular venous plasma Acta Endocrinologica 72:89–100
Wakeling D (1985) New moulting programme keeps water on and adds limestone Poultry World 25:8–10
Wilson SC and Sharp PJ (1973) Variations in plasma levels during the ovulatory cycle of the hen (Gallus domesticus) Journal of Reproduction and Fertility 35:561–564

T. M. Lovell et al.