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Interrelationship between feeding level and the metabolic hormones leptin, ghrelin and obestatin in control of chicken egg laying and release of ovarian hormones

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ABSTRACT

The aim of the present experiment is to examine the role of nutritional status, metabolic hormones and their interrelationships in the control of chicken ovarian ovulatory and secretory activity. For this purpose, we identified the effect of food restriction, administration of leptin, ghrelin 1-18, obestatin and combinations of food restriction with these hormones for 3 days on chicken ovulation (egg laying) rate and ovarian hormone release. The release of progesterone (P), testosterone (T), estradiol (E) and arginine-vasotocin (AVT) by isolated and cultured ovarian fragments was determined by EIA. It was observed that food restriction significantly reduced the egg-laying rate, T, E and AVT release and promoted P output by ovarian fragments. Leptin, administrated to *ad libitum*–fed chickens, did not change these parameters besides promoting E release. Nevertheless, administration of leptin was able to prevent the effect of food restriction on ovulation, T and E (but not P or AVT) release. Ghrelin 1-18 administration to *ad libitum*–fed birds did not affect the measured parameters besides a reduction in P release. Ghrelin 1-18 administration prevented the food restriction–induced decrease in ovarian T, E and AVT, but it did not change P output or egg laying. Obestatin administrated to control chicken promoted their ovarian P, E and inhibited ovarian AVT release but did not affect egg laying. It was able to promote the effect of food restriction on P, T and AVT, but not E release or egg laying. Our results (1) confirm an inhibitory effect of food restriction on chicken ovulation rate; (2) shows that food restriction–induced reduction in egg laying is associated with a decrease in ovarian T, E and AVT and an increase in ovarian P release; (3) confirm the involvement of metabolic hormones leptin, ghrelin and obestatin in the control of chicken ovarian hormones output; and (4) the ability of metabolic hormones to mimic/antagonize or prevent/promote the effects of food restriction on both egg laying and ovarian hormones demonstrates that nutritional status can influence ovarian ovulatory and endocrine functions via changes in metabolic hormones.

1. Introduction

The synchronisation of reproduction with optimal nutritional conditions is very important for species survival. It has been postulated that hypothetical mediators of nutrition could affect reproduction by (1) affecting nutrition, (2) controlling reproduction and (3) its changes could modify the effects of nutrition on reproduction. At the basis of these criteria, it is proposed that nutritional status affects mammalian reproductive processes via metabolic hormones—leptin, ghrelin and obestatin (see Navarro and Kaiser, 2013; Roa and Tena-Sempere, 2014; Sirotkin, 2014 for review). In birds, malnutrition inhibits ovarian follicullogenesis (Hocking, 2004) via induction of ovarian follicular cell apoptosis (Paczoska-Eliasiewicz et al., 2003) and affects metabolic hormones and their receptors (Paczoska-Eliasiewicz et al., 2003; Kaiya et al., 2007; Sirotkin et al., 2013). The involvement of leptin (Paczoska-Eliasiewicz et al., 2003, 2006; Sirotkin and Grossmann, 2007), ghrelin (Sirotkin et al., 2006; Sirotkin and Grossmann, 2008; Sirotkin et al., 2013) and obestatin (Sirotkin et al., 2013) in the control of chicken ovarian functions have been demonstrated. Finally, leptin administration during fasting was able to delay malnutrition-induced cessation of egg laying, attenuated the regression of ovarian follicles and abolished the fasting-induced apoptosis of ovarian follicular cells (Paczoska-Eliasiewicz et al., 2003). Despite the importance of such findings for understanding the endocrine mechanisms integrating nutrition and reproduction and for the improvement of farm avian reproduction,

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the mediátory roles of other metabolic hormones, ghrelin and obestatin, have not yet been demonstrated in this way. It is proposed that if these hormones mediate the effect of undernutrition, their administration could either prevent or promote the effect of food restriction.

The aim of the present experiments was to examine the role of nutritional status, metabolic hormones and their functional interrelationships in the control of chicken ovarian ovulatory and secretory activity, i.e. whether either food restriction or metabolic hormones affect ovarian functions, and whether metabolic hormones (hypothetical mediators of nutritional effect on reproduction) can modify ovarian response to food restriction. For this purpose, we identified the effect of food restriction, administration of leptin, ghrelin 1-18, obestatin and combinations of food restriction with these hormones on chicken ovulation (egg laying) rate and ovarian hormone release.

2. Materials and methods

2.1. Animal experiments, tissue collection and culture

Young (about 7 months of age) white leghorn hens (LSL), weight 1,1–1,2 kg with an egg-laying rate of more than 95%, were held in individual cages under standard conditions at the Experimental Station of the Institute of Animal Science on a 12 L:12 D photoperiod (illumination 08:00–20:00). Conditions of their care, manipulations and use corresponded with the instructions of EC no. 178/2002 and related EC documents, and they were approved by local ethics commission. After two months of adaptation to conditions in the experimental farms, the hens were divided into eight experimental groups: (1) control group was fed *ad libitum*, no hormone treatment; (2) group fed *ad libitum* and treated with i.m. injection of human recombinant leptin; (3) group fed *ad libitum* and treated with i.m. injection of human recombinant ghrelin 1-18. This trunicated ghrelin analogue ghrelin 1-18 mimicked the effect of full-length ghrelin 1-28 on chicken ovarian cells (Sirotkin and Grossmann, 2008); (4) group fed *ad libitum* and treated with i.m. injection of human recombinant obestatin; (5) group subjected to food restriction, no hormone treatment; (6) group subjected to food restriction and treated with i.m. injection of human recombinant leptin; (7) group subjected to food restriction and treated with i.m. injection of human recombinant ghrelin 1-18; (8) group subjected to food restriction, and treated with i.m. injection of human recombinant obestatin. Animals in the food-restricted groups had no access to food during the whole experiment, whilst all the animals have permanent access to drinking water. Hormonal treatments combined with food restriction were started together with food restriction. All research grade hormones were purchased from Peptides International Inc. (Louisville, Kentucky, USA). They were dissolved in sterile 0.7% NaCl immediately before the start of experiments and injected i.m. at doses 30 μg/animal in 1 ml of 0,7% NaCl, 6 times, every 10–12 hours, in the daytime (at 08:00 and 18:00). This dose is comparable with the amount of hormones in chicken organism and with treated doses reported previously (Paczoska-Eliasiewicz et al., 2003, 2006; Kaiya et al., 2007; Sirotkin et al., 2013). During the experiment, the egg-laying rate (number of eggs to number of animals) was calculated daily. The animals were killed by decapitation 1,5 hours after the last injection (between 09:00 and 11:00 am), and the largest (F1–F2) follicles were isolated from the ovary. The stage of folliculogenesis was determined by recording the time of the last oviposition and by the size and the position of the next ovarian follicle. Fragments of the follicular wall (5 mm in diameter, weight 24 $\pm$ 8 mg) were isolated as described previously (Sirotkin and Grossmann, 2003, 2006). After washing three times in sterile culture medium (DMEM/F-12 1:1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic–antimycotic solution; all from Sigma, St. Louis, USA), these fragments were cultured without treatments for 2 d in 2 ml culture medium in Falcon 24-well plates (Becton Dickinson, Lincoln Park, USA) at 38.5 °C under 5% CO_2 in humidified air. This protocol provides maximal accumulation of ovarian hormones into the culture medium and the most reliable characteristic of ovarian secretory activity (Sirotkin and Grossmann, 2003).

2.1.1. Immunoassay

Concentrations of progesterone (P), testosterone (T), estradiol (E) and arginine-vasotocin (AVT) were determined in 25 μl aliquots of incubation medium by EIA and RIA, previously validated for use in culture medium. These hormones are considered as the indexes of ovarian secretory activity, stress, response to hormonal stimuli and the key regulators of both mammalian (Sirotkin, 2014) and chicken (Sirotkin and Grossmann, 2006, 2007, 2008; Sirotkin, 2014) ovarian functions.

P concentrations were measured as described previously (Prakash et al., 1987). Rabbit antiserum against P was obtained from Research Institute for Animal Production "Schoonoord", Netherlands. It cross-reacted $\leq$0.1% with E_2, dihydrotestosterone, T and 17-beta hydroxyprogesterone. The sensitivity of the EIA was 12.5 pg/ml. Inter- and intra-assay coefficients of variation did not exceed 3.3% and 3.0%, respectively.

T was assayed according to Münster (1989) by using antisera against steroids (produced by the Institute of Animal Science, Neustadt, Germany). The sensitivity of the assay was 10 pg/ml. The cross-reactivity of the T antiserum was $\leq$96% to dihydrotestosterone, $\leq$3% to androstenedione, $\leq$0.01% to P_4 and E_2, $\leq$0.02% to cortisol and $\leq$0.001% to corticosterone. Inter- and intra-assay coefficients of variation were 12.3% and 6.8% respectively.

E concentrations were evaluated according to Münster (1989) by using antisera against steroids (produced by the Institute of Animal Science, Neustadt, Germany) with an assay sensitivity of 5 pg/ml. The cross-reactivity of the E_2 antiserum was $\leq$2% to estrone, $\leq$0.3% to estriol, $\leq$0.004% to T and $\leq$0.0001% to P_4 and cortisol. The inter- and intra-assay coefficients of variation did not exceed 16.6% and 11.7%, respectively.

AVT was determined by using RIA according to Gray and Simon (1983). The anti-AVT antiserum was kindly provided by Dr. D.A. Gray (Max-Plank Institute for Physiological and Clinical Research, Bad Nauheim, Germany), which cross-reacted $\leq$1.0% with mesotocin and angiotensin II. The sensitivity of the RIA is 0.3 pg/ml. Inter- and intra-assay coefficients of variation did not exceed 8.8% and 7.2%, respectively.

2.1.2. Statistics

The data shown are means of values obtained in three separate experiments performed on separate days using separate animals (8 animals per group) and their ovaries. The egg-laying rate was assessed by inspection of each individual cage two times a day (at 08:00 am and 17:00 pm). The egg-laying rate in the last day of the experiment was presented. In each in vitro experiment, each experimental group was composed of six culture wells with ovarian fragments. Assays of hormone levels in the incubation media were performed in duplicate. The values of blank controls were subtracted from the value determined by RIA/EIA in cell-conditioned medium to exclude any non-specific background (less than 15% of total values). The rates of substance secretion were calculated per mg tissue/day. Significant differences between the experiments were evaluated by using two-way ANOVA. When the effects of treatments were revealed, data from the experimental and control groups were compared by Chi-square test (percentage of egg laying) or Wilcoxon–Mann–Whitney multiple range test (release of hormones) using Sigma Plot 11.0 statistical software (Systat Software, GmbH, Erkrath, Germany). Differences from control at $P \leq 0.05$ were considered as significant.