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THE INVOLVEMENT OF LEPTIN, MAP KINASE- AND CDC2 KINASE-DEPENDENT INTRACELLULAR MECHANISMS IN THE CONTROL OF HORMONE RELEASE BY CHICKEN OVARIAN GRANULOSA CELLS

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ABSTRACT

The aim of our in-vitro experiments was to examine, whether the metabolic hormone leptin, mitogen activated kinase (MAPK) and cyclin-dependent protein kinase (CDK) can control secretory functions of avian ovarian cells and to outline, whether MAPK and CDK can be potential intracellular mediators of leptin effects. Granulosa cells isolated from chicken ovaries were cultured with leptin (0, 1, 10, 100, 1.000 or 10.000 ng/ml medium) in the presence or absence of MAPK blocker PD98059 and CDK blocker olomoucine. The release of hormones (arginine-vasotocin, AVT, estradiol, E_2 and progesterone, P_4) was evaluated by EIA and RIA. It was found that leptin inhibited release of AVT but stimulated E_2 and P_4 output. MAPK blocker, when given alone, reduced AVT release, but promoted secretion of E_2 and P_4. Moreover, it prevented leptin action on P_4, inverted leptin effect on E_2, but did not affect a pattern of leptin action on AVT. CDK blocker, when given alone, inhibited AVT and E_2 release, but stimulated P_4 output. Moreover, CDK blocker prevented or inverted leptin action on the release of all hormones studied. Present observations demonstrate that leptin, MAPK and CDK are involved in the control of chicken ovarian secretory activity (release of steroid and nonapeptide hormone), and leptin can affect ovarian hormones through MAPK- and CDK-dependent intracellular mechanisms.

Key words: Leptin, ovary, progesterone, testosterone, estradiol, arginine-vasotocin, mitogen-activated protein kinase (MAP kinase, MAPK), cyclin-dependent protein kinase (CDC2 kinase, CDK), chicken

INTRODUCTION

The metabolic hormone leptin, a product of adipose and some other tissues, can mediate effect of metabolic and nutrition factors on reproductive processes. Its production is increased under the influence of food intake and development of adipose tissue. Leptin can control reproductive processes through hypothalamic LH-RH and hypophyseal gonadotropins (Smith et al., 2002; Munoz-Gutierrez et al. 2005; Zieba et al., 2005), although direct effects of leptin on the ovary have been documented. Leptin, through ovarian receptors, can control the secretory activity of cultured rat, bovine, porcine, sheep and human ovarian cells (release of progesterone, P_4, testosterone, T, estradiol, E_2, prostaglandin, oxytocin, IGF-I and its binding proteins, Kitawaki et al., 1999; Almog et al., 2001; Duggal et al., 2002, Ruiz-Cortez et al., 2003, Munoz-Gutierrez et al., 2005, Sirotkin et al., 2005) and promote porcine oocyte maturation (Craig et al., 2004). Chicken could be an unique model for the study of ovarian cell proliferation and apoptosis, because of daily ovulation and high rate of proliferation, apoptosis, folliculogenesis and remodelling in this organ. Results of in-vivo experiments of Paczoska-Eliasiewicz et al. (2003, 2006) showed, that in this species leptin can advance sexual maturation, ovarian folliculogenesis, plasma LH and steroid hormone level and inhibit nuclear apoptosis in ovarian follicles (Paczoska-Eliasiewicz et al, 2003, 2006). Presence of leptin receptors in both chicken hypothalamus (Ohkubo

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et al., 2000, Paczoska-Eliasiewicz et al., 2003; Dridi et al., 2005) and ovary (Paczoska-Eliasiewicz et al., 2003; Cassy et al., 2004) and effect of leptin injections on chicken plasma LH (Paczoska-Eliasiewicz et al., 2003) indicates, that leptin in birds, like in mammals, might potentially affect reproduction at both hypothalamic and gonadal level. In isolated chicken ovarian cells leptin was able to control proliferation, apoptosis, P_4, T, E_2 and arginine-vasotocin (AVT), an analogue of mammalian nonapeptide hormones oxytocin and vasopressin (Sirotkin and Grossmann, 2007a) indicating direct control of avian ovarian functions by leptin.

Avian, as well as mammalian ovarian functions are under control of intracellular protein kinases – protein kinase A (PKA), mitogen-activated protein kinase (MAPK), cyclin-dependent kinase (CDK) a.o., which could mediate action of some peptide hormones (Sirotkin and Grossmann, 2006, 2007b). It was demonstrated, that in mammalian ovarian tissue leptin could act through cAMP/PKA- and MAPK-dependent intracellular mechanism, which could be involved in mediating leptin action on porcine oocytes (Craig et al., 2004), human (Choi et al., 2005) and rabbit (Zerani et al., 2004) ovarian follicular cells. In chicken ovarian cells leptin was able to affect the accumulation of PKA, MAPK, and CDK suggesting that these protein kinases could be potential mediators of leptin action on avian ovaries (Sirotkin and Grossmann, 2007a). There is however no direct evidence for the role of these protein kinases in mediating leptin action on avian ovarian cells. Such direct evidence could be the ability of protein kinase blockers to prevent or to promote leptin action. Such studies have not been performed yet.

The aim of our in-vitro experiments was to examine, (1) whether leptin can directly control the release of peptide and steroid hormones by chicken ovarian granulosa cells, (2) whether MAPK and CDK are involved in control of these processes, and (3) whether MAPK and CDK can be involved in mediating leptin effects on ovarian hormones. For these purposes we analysed the release of AVT, P_4 and E_2 by granulosa cells isolated from chicken ovaries and cultured with or without addition of leptin, MAPK blocker, CDK blocker and with their combination.

MATERIAL AND METHODS

Preparation, culture and processing of ovarian cells

Ovaries were collected from White Leghorn hens (LSL) about 6 months old, with an egg laying rate of more than 95%. Animals were kept under standard conditions at the Experimental Station of the Institute of Animal Science on a photoperiod 12L:12D (illumination 8.00a. m.-8.00p.m.). Experiments were conducted in agreement with the guidelines of the European Group of Advisers on the ethical Implications of Biotechnology (1991-1997), the European Group on Ethics in Science and New Technologies (as for 1998), and the corresponding German and Slovak ethical regulations. The studies were approved by the committee of animal care of the Institute of Animal Science. Birds were decapitated between 9.00 and 11.00 a.m. 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 follicular wall were isolated as described previously (Sirotkin and Grossmann, 2003, 2006). After three times washing 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 with preparations for 4 days 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 and a good response to hormones (Sirotkin and Grossmann, 2003).

Ovarian follicles were washed 3 times in sterile solution of 0.7% NaCl. Granulosa cells were gently scraped from the inner surface of follicular wall by a lancet and washed 3 times by centrifugation and resuspension 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). Thereafter they were cultured in 0.3 ml of medium in Lab-Tek chamber- slides (Nunc Inc., Naperville, USA) at 10^6 cells/ml (determined by haemocytometer) at 38.5°C under 5% CO_2 in humidified air. After 4 days of pre-culture (when the cells reached 50-60% confluent monolayer), the medium was replaced with a fresh medium with or without preparation, and the cells were cultured in fresh medium for 2 days.

Treatments included recombinant human leptin of research grade (Sigma, at doses 0, 1, 10, 100, 1.000 or 10.000 ng/ml), PD98059 (MAPK blocker; Calbiochem-Novabiochem Corp., La Jolla, Ca., USA, 100 ng/ml) or olomoucine (CDK blocker; Calbiochem-Novabiochem Corp., 1 µg/ml) used separately or in combination with leptin. Previous studies performed on chicken ovarian cells (Sirotkin and Grossmann, 2006, 2007b) showed, that leptin and these protein kinase blockers are effective at the concentrations and time of treatment used. Leptin preparation was dissolved in culture medium immediately before the experiment. Protein kinase blockers were prepared at 1 mg/ml in 50 µl of DMSO and were dissolved in culture medium immediately before the experiment, so that the final concentration of DMSO did not exceed 0.001 %. Controls intended for RIA/EIA included not only granulosa cells cultured without treatments, but

also incubation medium cultured without ovarian tissue (blank assay).

Immediately after culture the medium conditioned by granulosa cells was gently aspirated from culture wells and frozen at –18°C until assay by RIA or EIA.

RIA and EIA

Levels of P_4, E_2 and AVT were determined in 25-100 µl of incubation medium by RIA and EIA, previously validated for use in culture medium, using antisera against steroids produced in the Institute of Animal Science, Neustadt, Germany and against AVT produced in the Max-Plank Institute for Physiological and Clinical Research, Bad Nauheim, Germany. P_4 concentrations were measured using EIA as described previously (Prakash et al., 1987). Rabbit antiserum against P_4 was obtained from Research Institute for Animal Production, Schoonoord, Netherlands. It cross-reacts with estradiol, dihydrotestosterone, testosterone and 17-beta hydroxyprogesterone at ≤0.1%. The sensitivity was 12.5 pg/ml. Inter- and intraassay coefficients of variation did not exceed 3.3% and 3.0%, respectively. E_2 concentrations were evaluated using EIA according to Münster (1989). The sensitivity was 5 pg/ml. The cross-reactivity of the E_2 antiserum was < 2% to estrone, ≤ 0.3% to estriol, ≤ 0.004% to testosterone and ≤ 0.0001% to P_4 and cortisol. Inter- and intraassay coefficients of variation did not exceed 16.6% and 11.7%, respectively. AVT was determined using RIA according to Gray and Simon (1983). The sensitivity was 0.3 pg/ml. The antiserum cross-reacted with mesotocin and angiotensin II at ≤1.0%. Inter- and intra-assay coefficients of variation did not exceed 8.8% and 7.2%, respectively.

Statistics

The data shown are means of values obtained in three separate experiments performed on separate days using separate pools of ovaries, each obtained from 10-12 animals. At each in-vitro experiment each experimental group was composed of six culture wells. Assays of hormone levels in the incubation media were performed in duplicate. The samples intended for RIA/EIA were processed separately. The values of blank control 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 10^6 cells/day. Significant differences between experiments were evaluated using two-way ANOVA. When effects of treatments were revealed, data from the experimental and control groups were compared by Wilcoxon-Mann-Whitney multiple range test. Differences from control at $P < 0.05$ were considered as significant.

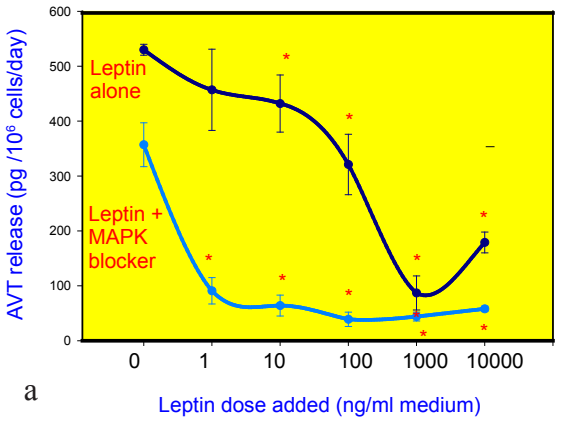
RESULTS AND DISCUSSION

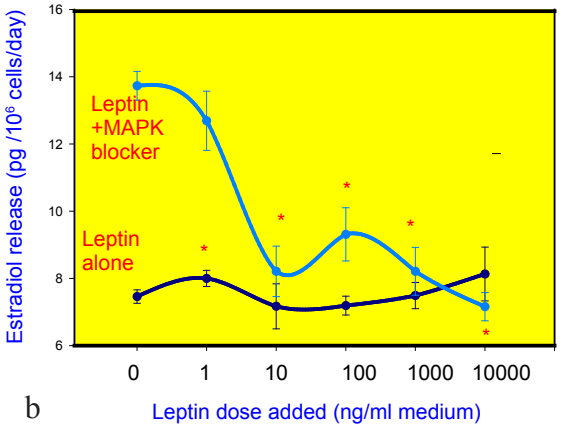
Isolated granulosa cells were able to survive in culture and to secrete hormones. RIA/EIA demonstrated the release of P_4, E_2 and AVT by these cells into the culture medium (Fig.1,2).

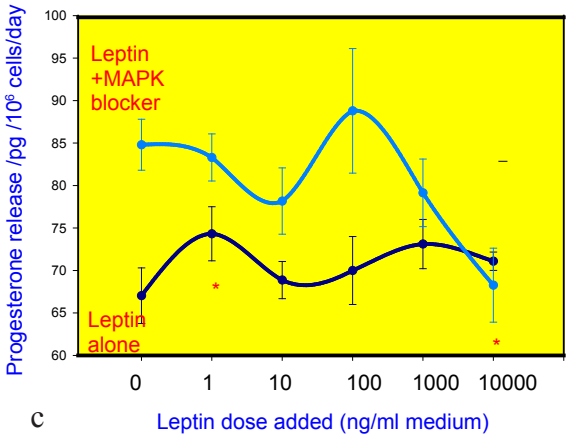
Secretory activity of cells was influenced by additions of leptin and protein kinase blockers. Leptin significantly decreased the release of AVT (at doses 10-10.000 ng/ml), but increased E_2 and P_4 (both at dose of 1 ng/ml, Fig.1,2). MAPK blocker PD98059, when given alone, reduced AVT release, but promoted secretion of E_2 and P_4. Moreover, it prevented stimulatory action of leptin on P_4, inverted stimulatory effect of leptin on E_2 to the inhibitory ones, but it did not affect a pattern of leptin action on AVT (Fig.1). CDK blocker olomoucine, when given alone, inhibited AVT and E_2 release, but stimulated P_4 output. Moreover, CDK blocker prevented inhibitory action of leptin on AVT and inverted action of leptin on the release of steroid hormones ~~studied~~ from the stimulatory to the inhibitory ones (Fig.2).

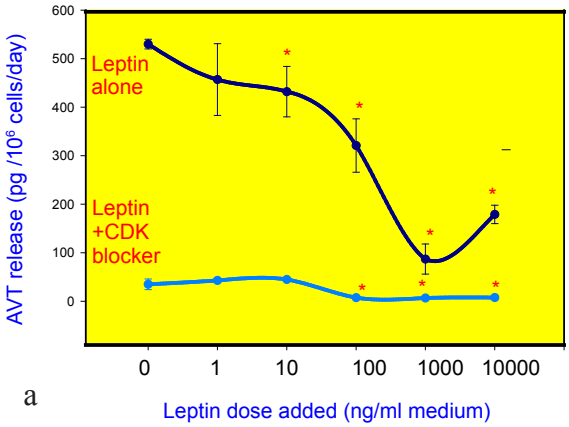
Our results correspond to the previous reports (Paczoska-Eliasiewicz et al., 2003, 2006, Sirotklin and Grossmann, 2007a) on the influence of leptin on the release of steroid hormones by chicken ovarian cells. It is possible, that some observed changes are not a primary, but a secondary. For example, increased release of E_2 after leptin addition could be due to changes in the production of its precursor, P_4. Furthermore, results of present experiments demonstrate, for the first time, the inhibitory influence of leptin on ovarian AVT release.

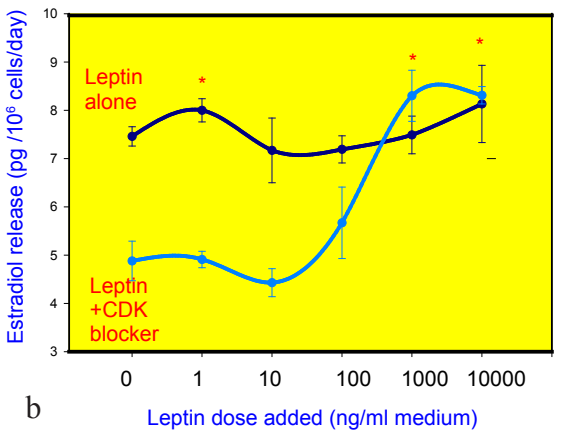
Present observations confirm our previous data (Sirotkin and Grossmann, 2006, 2007b) on the ability of MAPK and CDK blockers to affect chicken ovarian secretory activity. All these data suggest an importance of MAPK- and CDK-dependent intracellular mechanisms in the control of ovarian steroid and nonapeptide hormone release. It was postulated, that MAPK and CDK can not be the only regulators of ovarian functions, but also mediators of effects of some hormones on ovarian cells. To be such mediators, (1) MAPK and CDK should be controlled by hormones, and (2) blockers of MAPK and CDK should prevent, promote or invert hormone action. Using this approaches it was suggested (Sirotkin and Grossmann, 2006, 2007b), that MAPK and CDK can mediate action on IGF-II and ghrelin on chicken ovarian cells. Previously we demonstrated, that these protein kinases could be regulated by leptin too (Sirotkin and Grossmann, 2007a). Our present results demonstrate the ability of MAPK and CDK blockers to prevent and even invert leptin action on chicken ovarian cells. This could be the first direct evidence, that leptin affects chicken ovarian secretory activity through MAPK- and CDK-dependent intracellular mechanisms.











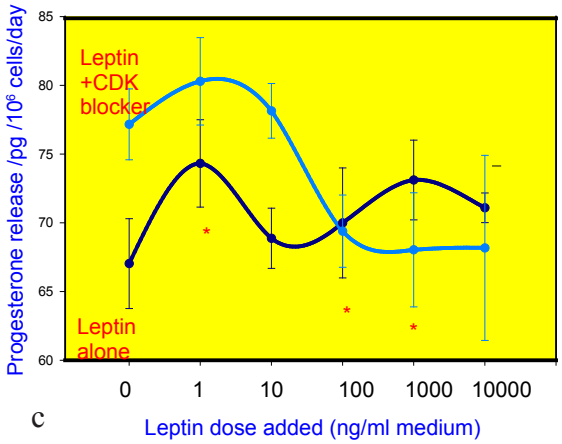


Fig. 1: **Effect of leptin (0, 1, 10, 100, 1.000 or 10.000 ng/ml medium) with and without presence of mitogen activated protein kinase blocker, PD98059, on the release of arginine-vasotocin (a), estradiol (b) and progesterone (c) by cultured chicken ovarian granulosa cells. Values were obtained by combination of RIA/EIA as indicated in Materials and Methods. Values are means + S.E.M. * P < 0.05 vs. control group (medium without leptin)**

Fig. 2: **Effect of leptin (0, 1, 10, 100, 1.000 or 10.000 ng/ml medium) with and without presence of cyclin-dependent protein kinase blocker, olomoucine, on the release of arginine-vasotocin (a), estradiol (b) and progesterone (c) by cultured chicken ovarian granulosa cells. Values were obtained by combination of RIA/EIA as indicated in Materials and Methods. Values are means + S.E.M. * P < 0.05 vs. control group (medium without leptin)**

Taken together, present results demonstrate that leptin, MAPK and CDK are involved in the control of chicken ovarian secretory activity (release of steroids and nonapeptide hormones), and leptin can affect ovarian hormones through MAPK- and CDK-dependent intracellular mechanisms.

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