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Independent, Non-IGF-I Mediated, GH Action on Estradiol Secretion by Prehierarchical Ovarian Follicles in Chicken. *In vitro* Study*

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Information concerning the role of growth hormone (GH) in the local regulation of ovarian activity in birds is limited. Therefore, the aim of the present study was to determine whether in the domestic hen GH influences *in vitro* estradiol secretion by prehierarchical ovarian follicles. Moreover, the interaction between GH and IGF-I on estradiol secretion was examined. Small white (1-4 mm), large white (4-6 mm) and yellowish (6-8 mm) ovarian follicles were isolated at the stage of 2 h after ovulation. In the first experiment (n=8 hens), whole follicles (small white, n=6/dose/ovary; large white, n=1/dose/ovary and yellowish, n=1/dose/ovary) were incubated for 24 h at 38°C in a medium supplemented with 0 (control), 1, 10 or 100 ng/ml of chicken GH (cGH). In the second experiment (n=6 hens), follicles were incubated in the same way in a medium with 0 (control), 10 ng/ml cGH, 25 ng/ml human IGF-I or cGH+hIGF-I (10 ng/ml+25 ng/ml). Following incubation the estradiol concentration was determined in media (RIA) and protein in the tissues of the follicular wall (Lowry). The secretion of estradiol was expressed per milligram of protein. The experiments revealed that both cGH and hIGF-I stimulated estradiol secretion by examined chicken ovarian follicles. The simultaneous addition of cGH and hIGF-I increased estradiol secretion by ovarian follicles as compare to the control. These hormones added together did not have an additive effect when compared to their separate actions. The results obtained suggest that both GH and IGF-I are important stimulators of estradiol production in chicken nonhierarchical ovarian follicles. We propose independent, non-IGF-I-mediated GH action on estradiol secretion.

Key words: GH, IGF-I, estradiol secretion, ovary, chicken.

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In the ovary of a laying hen the growing follicles are represented by numerous prehierarchical white and yellowish follicles with a diameter >1-8 mm and five to seven yellow preovulatory ones with a diameter >8-36 mm arranged in a size hierarchy. Steroidogenic activity of follicles changes during their growth and maturation. In white and yellowish follicles the granulosa layer is steroidogenically incompetent whereas the theca is the source of ovarian estradiol. In yellow follicles both theca and granulosa layer are steroidogenically active and theca is a source of estradiol while granulosa is a source of progesterone (HRABIA *et al.* 2004; RZĄSA *et al.* 2009). The regulation of ovarian steroidogenesis is multihormonal. In addition to pituitary-derived gonadotropins, which are essential for steroid production, the locally synthesised regulators such as growth factors (see review ONAGBESAN *et al.* 2009), biogenic amines (RZĄSA & PACZOSKA-ELIASIEWICZ 2000) and steroids (HRABIA *et al.* 2008a) are also involved in this process. In the last few years the presence of growth hormone (GH) (HRABIA *et al.* 2008b) and its receptors (HECK *et al.* 2003; LEBEDEVA *et al.* 2004; HRABIA *et al.* 2008b) has been documented in the chicken ovary, which suggests that this organ is a target site for endocrine and autocrine/paracrine actions of GH.

In vertebrates GH exerts multiple effects on diverse physiological processes. Among them there is a regulation of reproductive functions (see review HULL & HARVEY 2000, 2001; BACHELOT *et al.* 2002; MEINHARDT & HO 2006; ROUSSEAU & DUFOUR 2007). At the cellular level, GH activities include gene transcription, mitogenesis, cytoskeletal reorganization and cell migration (see re-

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view PILECKA *et al.* 2007). In birds, participation of GH in the reproductive functions is poorly known and its role in the local regulation of ovarian activity has had little attention. Our recent study showed that injections of exogenous chicken GH (cGH) alter steroid content, proliferation and apoptosis in the chicken ovary during sexual maturation (HRABIA *et al.* 2011). Moreover, a previous experiment by Williams *et al.* (WILLIAMS *et al.* 1992) had evidenced an increase in the number of small ovarian follicles after GH administration to the laying hens. However, the role of GH in the chicken ovarian steroidogenesis is not elucidated. Accordingly, in the present investigation, the effect of GH on estradiol secretion by isolated chicken prehierarchical (white and yellowish) ovarian follicles has been examined.

It is well known that some actions of GH are mediated by insulin-like growth factors (IGF-I, IGF-II) which are produced in many tissues in response to GH (YOSHIMURA *et al.* 1994). In the chicken ovary all members of the IGFs system have been identified and the effect of IGFs on steroid secretion has been shown (see review ONAGBESAN *et al.* 2009). There are suggestions that IGFs are the local mediators of GH in the chicken ovary (ONAGBESAN *et al.* 2009). Therefore, in the present study an interaction between GH and IGF-I on *in vitro* estradiol secretion by chicken nonhierarchical ovarian follicles has also been assessed.

Material and Methods

All procedures were performed in accordance with the research protocols approved by the Local Animal Ethics Committee in Kraków, Poland (No. 50/OP/2004).

Animals

Experiments were carried out on Hy-Line laying hens (n=14) at the age of 27 (Exp. 1) or 30 (Exp. 2) weeks, caged individually under a photoperiod of 14L:10D (light on at 0800 h) with free access to food and water. Time of oviposition was recorded daily at 15 min intervals between 0800 h and 1500 h, and once at 1700 h. Birds used in the experiments, characterized by regular sequences of at least 20 eggs per clutch were decapitated 2 h after ovulation. From the ovaries the following prehierarchical follicles were isolated: small white (1-4 mm in diameter), large white (4-6 mm) and yellowish (6-8 mm).

Incubation procedure

In the first experiment (n=8 chickens), to examine the effect of increasing doses of GH on estradiol secretion, whole follicles were randomly assigned to 1 ml of Eagle's medium supplemented with recombinant chicken GH at a dose of 0 (control), 1, 10 or 100 ng/ml and 0.05 g/ml BSA and 2 μl/ml antibiotic-antimycotic solution (10000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B/ml). From each ovary either small white follicles (n=6) pooled together, large white follicle (n=1) or yellowish follicle (n=1) were incubated in a 24-well multidish at 38^{o}C for 24 h at each dose level.

In a separate experiment (n=6 chickens), to investigate the interaction between GH and IGF-I on estradiol secretion, follicles were incubated for 24 h in a medium without hormone (control), or a medium supplemented with 10 ng/ml of cGH, 25 ng/ml of human IGF-I, or cGH combined with hIGF-I at the above concentrations. The dose of cGH was established on the basis of the results of the first experiment and the dose of hIGF-I was chosen according to Onagbesan *et al.* (ONAGBESAN *et al.* 1999).

Following incubation, both in Exp. 1 and Exp. 2 the medium was collected for estradiol determination and tissues of the follicular wall for protein determination by Lowry's method. The secretion of estradiol was expressed per milligram of protein.

Estradiol assay

Estradiol concentration in the medium was measured radioimmunologically using Spectria kits (Orion Diagnostica, Finland). The detection limit was 5.45 pg/ml and the mean recovery 99.5%. The cross reactivities of estradiol antiserum for estradiol, estron, and estriol were as follows: 100%, 0.97%, and 0.44%. The intra- and inter-assay coefficients of variation were 5.7% and 6.4%, respectively.

Statistical analysis

Data were analysed statistically by one-way ANOVA followed by Duncan's multiple range test. Values are expressed as the mean ± SEM from 8 (Exp. 1) or 6 (Exp. 2) ovaries and are considered significantly different at $P<0.05$.

Results

Effect of cGH on *in vitro* estradiol secretion by whole prehierarchical ovarian follicles (Exp. 1)

Secretion of estradiol expressed per mg of protein in the control group was 356 ± 38, 425 ± 51 and 268 ± 30 pg/mg protein/24 h by small white, large white and yellowish follicles, respectively (Fig. 1). cGH at doses of 1 ng and 10 ng/ml significantly increased estradiol secretion by all examined follicles (42-67% and 39-65%, respectively) and

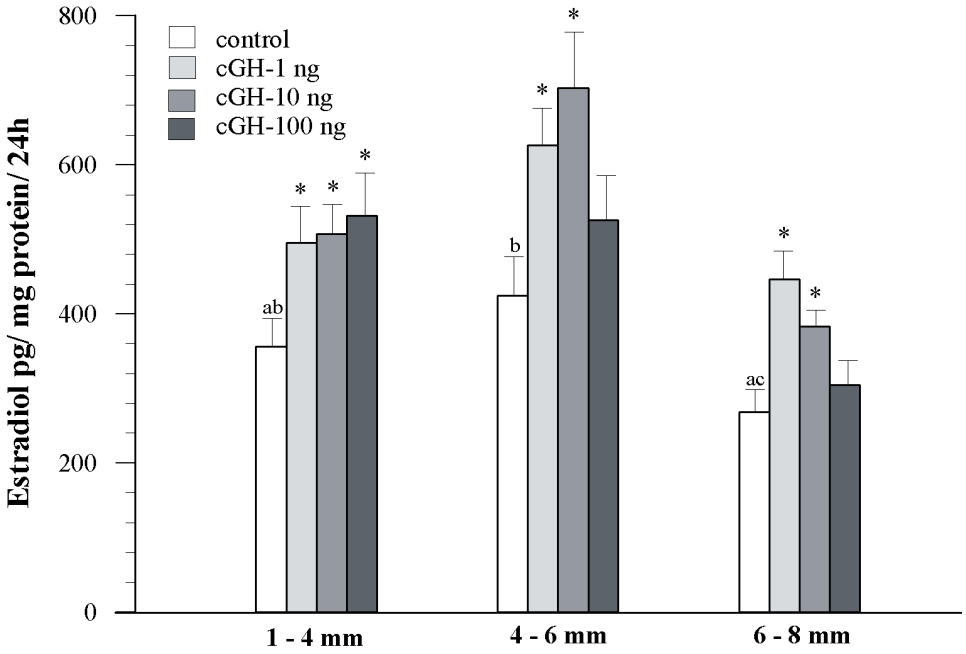

Fig. 1. Effect of increased concentrations (1, 10 and 100 ng/ml) of cGH on *in vitro* estradiol secretion (pg/mg protein/24h) by whole small white (1-4 mm), large white (4-6 mm) and yellowish (6-8 mm) ovarian follicles.
Each value represents the mean ± SEM from 8 chickens. Means marked with different letters within the control group are significantly different at P<0.05. *P<0.05 .

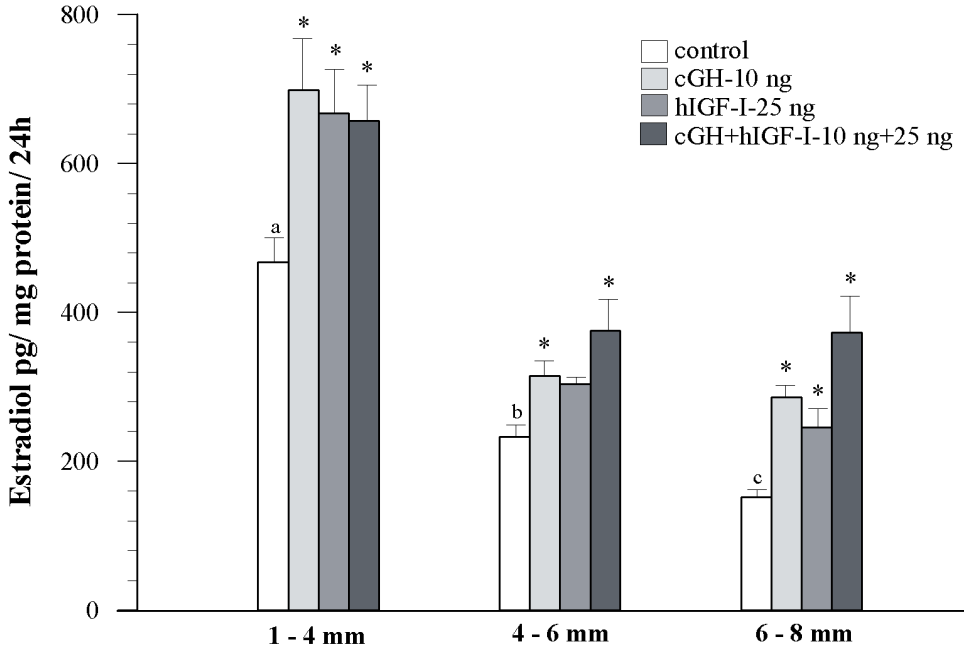

Fig. 2. The combined effect of cGH (10 ng/ml) and hIGF-I (25 ng/ml) on *in vitro* estradiol secretion (pg/mg protein/24 h) by whole small white (1-4 mm), large white (4-6 mm) and yellowish (6-8 mm) ovarian follicles.
Each value represents the mean ± SEM from 6 chickens. Means marked with different letters within the control group are significantly different at P<0.05. *P<0.05.

at a dose of 100 ng/ml significantly elevated estradiol secretion by small white follicles (49%) (Fig. 1).

Effect of hIGF-I on cGH-stimulated *in vitro* estradiol secretion by whole prehierarchical ovarian follicles (Exp. 2)

Under the control conditions the highest secretion of estradiol (pg/mg protein/24 h) was by small white follicles (468 ± 33) and the lowest was by yellowish follicles (152 ± 10) (Fig. 2). The addition of cGH into the culture medium significantly

increased basal estradiol secretion by each class of examined follicles: small white by 49%, large white by 34% and yellowish by 89.6%. hIGF-I increased estradiol secretion by small white follicles by 43% and yellowish follicles by 62%. Treatment of the follicles with a combination of cGH+hIGF-I significantly stimulated estradiol secretion by each group of the follicles: 40%, 61% and 146%, respectively for small white, large white and yellowish in comparison to control. As compared to individual effects of cGH and hIGF-I, combined treatment with cGH+hIGF-I did not intensify estradiol secretion by examined ovarian follicles (Fig. 2).

Discussion

The results of the present investigation reveal that *in vitro* estradiol secretion expressed per mg of protein by the prehierarchical follicles (1-8 mm) decreases during follicular growth. These data are consistent with previous findings (HRABIA *et al.* 2004; LEE & BAHR 1994) and correlate with changes in the examined steroid level in the wall of follicles (RZĄSA *et al.* 2002).

The first finding of the present study was that all used doses of cGH were effective and had stimulatory action on estradiol secretion by nonhierarchical follicles. The doses of 1 ng/ml and 10 ng/ml of cGH increased estradiol secretion by all groups of follicles. The highest dose of cGH, i.e. 100 ng/ml, elevated estradiol secretion by small and large white follicles. The stimulating effect of cGH on estradiol secretion observed in this work is in agreement with the previously observed effect of cGH in *in vivo* study. Namely, cGH administration to the chickens during sexual maturation increased concentrations of estradiol in the ovary (HRABIA *et al.* 2011). Similarly, our observations are in line with *in vitro* effects of GH on mammalian ovarian cells reported previously (OVESEN *et al.* 1994; YOSHIMURA *et al.* 1993b; GREGORASZCZUK *et al.* 2000; KOŁODZIEJCZYK *et al.* 2003), although an insignificant or even inhibitory effect of GH on estradiol secretion was also noted (RAJKUMAR *et al.* 1993; SPICER & ECHTERNKAMP 1995). Hence, these observations indicate that the effect of GH on ovarian steroidogenesis may be dependent on follicle size (see review HULL & HARVEY 2001).

Since GH may influence ovarian function indirectly, at least in part, by stimulation of IGF-I production (YOSHIMURA *et al.* 1994), the effect of IGF-I on basal and cGH-stimulated estradiol secretion by nonhierarchical follicles of the chicken was also examined in this study. It was found that both cGH and hIGF-I act as stimulatory factors on estradiol secretion by chicken prehierarchical follicles. Contrary to the present authors' observations in white nonhierarchical follicles, Onagbesan *et al*. (ONAGBESAN *et al.* 1994, 1999) showed an inhibiting effect of IGF-I on estradiol secretion by theca cells of yellow hierarchical follicles in chickens. The opposite effect of IGF-I could be a result of different sizes and the maturational stage of the follicles. Some studies performed *in vitro* on porcine ovarian cells revealed stimulatory action of IGF-I on estradiol secretion (KOŁODZIEJCZYK *et al* 2001, 2003; GREGORASZCZUK *et al.* 2007).

The most important finding of the present investigation was that cGH and hIGF-I added together into the incubation medium had no additive effect on estradiol secretion by prehierarchical follicles. The lack of interaction between these hormones suggests that they act on chicken prehierarchical follicles directly by their own receptors. The IGF-I-independent mechanism of GH action in the ovary was also strongly suggested in the mammalian ovary (see review HULL & HARVEY 2001; BACHELOT *et al.* 2002).

The GH and IGF-I steroidogenic action might be associated with their effects on the synthesis and/or activity of key enzymes responsible for estradiol synthesis, including aromatase. To our knowledge there are no data indicating GH effects on aromatase activity in the small ovarian follicles in avian species, whereas an inhibiting action of IGF-I on both basal and LH-induced aromatase activity was revealed (ONAGBESAN *et al.*1994). On the other hand, GH stimulated the activity of aromatase, for instance, in the ovaries of women (TAPANAINEN *et al.* 1992), pigs (RAK *et al.* 2008) and sea trout (SINGH & THOMAS 1993). It was also observed that IGF-I present in a culture medium stimulated aromatase activity and gene expression in vitellogenic follicles in carp (PAUL *et al.* 2010).

The stimulating estradiol secretion action of GH and IGF-I may also reflect its induction of cell proliferation. We have previously reported that GH exhibits proliferating activity in the chicken small ovarian follicles which predominantly produce estradiol (HRABIA *et al.* 2011). On the other hand, the stimulating proliferation effect of IGF-I in the chicken ovary is well documented (see review ONAGBESAN *et al.* 2009).

Taking into consideration the results of the current study and our former data (HRABIA *et al*. 2011) we suggest that both GH and IGF-I are important stimulators of estradiol production in chicken nonhierarchical ovarian follicles. We propose an independent, non-IGF-I-mediated GH action on estradiol secretion.

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