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EFFECT OF SHORT-TERM FEED WITHDRAWAL ON GROWTH HORMONE RECEPTOR AND AROMATASE GENE EXPRESSIONS IN THE OVARY OF GROWING CHICKEN

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To evaluate the relationship between growth hormone (GH) action and estrogen synthesis in the chicken ovary during food intake disturbance, the expression of GH receptor (GHR) and aromatase mRNAs in the ovary of sexually immature chicken has been examined after short-term starvation. The ovaries were taken from the control (fed *ad libitum*; n=5), fasted for 24h (n=5) and fasted for 24h+re-fed for 24h (n=5) hens, and the ovarian stroma with primordial follicles and white follicles were isolated. The expression of the examined genes was determined in the ovarian tissues by semi-quantitative RT-PCR. It was found that: (1) GHR and aromatase mRNAs were present in the ovary of immature chickens, (2) the relative level of the GHR gene depended on the ovarian tissues, (3) the fasting caused a significant decrease in GHR and aromatase mRNA expressions in white follicles, and (4) the re-feeding caused an increase in the expression of both the examined genes in white follicles as compared with the fasted chicken. In conclusion, the results obtained indicate that short-term fasting affects GHR and aromatase mRNA expressions in white follicles of immature chickens. Simultaneous changes in the mRNA levels of the examined genes may suggest an involvement of GH in the regulation of aromatase gene expression in the developing ovary of chickens. On the other hand, a decrease in estradiol synthesis may affect GHR expression and GH action within the ovary.

Key words: GHR, aromatase, fasting, ovary, chicken

INTRODUCTION

A large body of evidence indicates that in female domestic birds the development and activity of the ovary are influenced by environmental factors, particularly by the feeding level, which in turn affects the metabolism rate. Changes in the level of circulating metabolites and hormones are the signals which link the metabolic status to the activation of the reproductive system (SUN et al., 2006; SWENNEN et al., 2007). One of the key regulators of metabolism is the growth hormone (GH). Recently, it has been shown that GH is expressed on mRNA and protein levels in all tissues of the chicken ovary before and after the onset of egg laying (HRABIA et al., 2008; AHUMADA-SOLÓRZANO et al., 2012) and GH receptors are similarly expressed in the chicken ovary (HECK et al., 2003; LEBE-

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DEVA et al., 2004; HRABIA et al., 2008). Moreover, the administration of GH stimulated the ovarian weight and the number of ovarian follicles as well as increased cell proliferation and decreased cell apoptosis in the ovary before the first oviposition (HRABIA et al., 2011). The studies have also revealed elevated levels of progesterone and estradiol in the chicken ovary around the time of chicken puberty after GH-treatment during maturation (HRABIA et al., 2011). In vitro studies have shown that GH stimulates in a dose-dependent manner the synthesis of progesterone in the primary granulosa cell culture and the mRNA expression of cholesterol side-chain cleavage enzyme (cytochrome P450scc) in yellow preovulatory follicles (AHUMADA-SOLÓRZANO et al., 2012). In addition, recent in vitro study of HRABIA et al. (2012) has demonstrated a stimulatory effect of GH on estradiol secretion by white and yellowish prehierarchical follicles which produce primarily ovarian estrogens in chickens (HUANG et al., 1979; RZĄSA et al., 2002; HRABIA et al., 2004a).

Previously, it has been found that during long-term starvation the concentration of circulating and ovarian estradiol decreases (PROSZKOWIEC and RZĄSA, 2001; PROSZKOWIEC-WĘGLARZ et al., 2005). On the other hand, the plasma GH level increases during food and dietary protein restriction (SWENNEN et al., 2007). However, in the available literature there are no data as to the relation between GH action and estradiol synthesis in the chicken ovary in the situation of food intake disturbance. Therefore, in the present study the effect of short-term starvation on the mRNA expression of GH receptor (GHR) and aromatase, a key enzyme in estrogen synthesis, in the ovary of sexually immature chicken has been examined.

MATERIALS AND METHODS

Animals

The experiment was carried out on growing Hy-Line Brown chickens (layer strain; n=15) caged individually under a photoperiod of 14L:10D. The experimental design was as described previously (HRABIA and JANKOŚ, 2011). Briefly, the hens at 15 weeks of age were divided into 3 equal groups: (i) fed ad libitum, (ii) fasted for 24h, and (iii) fasted for 24h+re-fed for 24h. After the indicated time the birds were decapitated; the ovaries were isolated and the ovarian stroma with primordial follicles (<1 mm in diameter) and white follicles (1-4 mm) present in the ovaries were collected. After removal of the existing yolk from the follicles, the tissues were quickly placed in RNAlater and stored at -20^{0}C until RNA extraction.

Total RNA isolation and RT-PCR analysis

Total RNA in ovarian tissues was extracted with TRI-reagent according to the manufacturer's recommendations (MRC, Inc., USA). Two µg of total RNAs of each tissue were reverse-transcribed with RevertAid M-MuLV reverse transcriptase (200 U; Fermentas, Lithuania) and oligo-dT_{18} primers (IBB, Warszawa, Poland). The obtained cDNAs were used for PCR amplifications carried out according to HRABIA et al. (2008) for GHR, SAKIMURA et al. (2002) for aromatase (CYP19), and HRABIA et al. (2004b) for ribosomal protein S17 (internal control). PCRs were performed in a Thermocycler Gradient (Eppendorf, Germany) in 12.5 µl of reaction mixture containing 1.25 µl of buffer (100 mmol Tris-HCl, pH 8.8, 500 mmol KCl, 0.8% No-

Table 1. Characteristic of primers and PCR conditions used in this study.

Gene	GenBank	Primer sequence	PCR product	PCR conditions
aromatase (CYP19)	J04047	F: 5'-CAGCCAGTTGTGGACTTAAT-3' R: 5'-CCTCTTCCTTTCATTGTCTG-3'	302 bp	95°C 30s, 56°C 30s, 72°C 30s, 27 cycles
GHR	47604939	F: 5'-CAGACAGCACTGACTCAGCTA-3' R: 5'-TGGTAAGGCTTTCTGTGGTGA-3'	321 bp	94°C 30s, 60°C 60s, 72°C 60s, 30 cycles
S17	AY215074	F: 5'-GAGAGCGCCTCGCGGCGTTT-3' R: 5'-GGCGCGGGTGATCATCGAGAA-3'	370 bp	94°C 20s, 60°C 20s, 72°C 30s, 25 cycles

nidet P40), 0.312 unit pol Taq DNA polymerase (Fermentas, Lithuania), 0.2 µmol sense and antisense primers (IBB, Warszawa, Poland), 0.2 mmol each dNTP, 1.5 mmol $MgCl_2$, and water (Fermentas, Lithuenia). The applied amplification profiles and the primers for GHR, aromatase and S17 are described in Table 1. The negative control (water) was included in all the reactions. PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide in 0.5x TBE buffer and the gels were photographed under UV light. The mRNA expression levels of the examined genes were normalized with ribosomal protein S17 mRNA. The averages obtained from five abundance determinations were used for statistical analysis.

Statistical analysis

The data were analyzed by two-way analysis of variance followed by Duncan's multiple-range test. The significance of differences was considered at the level of p<0.05. The results were expressed as the mean ± SEM of 5 birds.

RESULTS

The presence of GHR and aromatase mRNAs was found in the ovarian stroma and white follicles of immature chickens. As an amplification product there was a band with the expected size of 321 bp for GHR cDNA, 302 bp for CYP19 cDNA and 370 bp for S17 cDNA (Fig. 1).

The relative expression of GHR mRNA was higher in the ovarian stroma than in the white follicles present in the ovary. In the stroma of all the examined groups the expression of GHR mRNA was similar and ranged from 1.89 ± 0.058 to 2.08 ± 0.059. In white follicles the expression of GHR mRNA was significantly higher in the control than in the fasted chickens (1.77 ± 0.092 vs 1.43 ± 0.022). Re-feeding caused an increase in GHR mRNA expression to 1.53 ± 0.085 (Fig. 2).

The level of relative expression of aromatase mRNA in the stroma and white follicles was on the same level and ranged from 1.74 ± 0.047 to 2.31 ± 0.017. Short-term starvation did not change significantly the expression of aromatase mRNA in the stroma, while in white follicles it caused a decrease from 2.19 ± 0.149 to 1.73 ± 0.047. After re-feeding, the level of aromatase mRNA expression returned to the control value and was 2.31 ± 0.017 (Fig. 3).

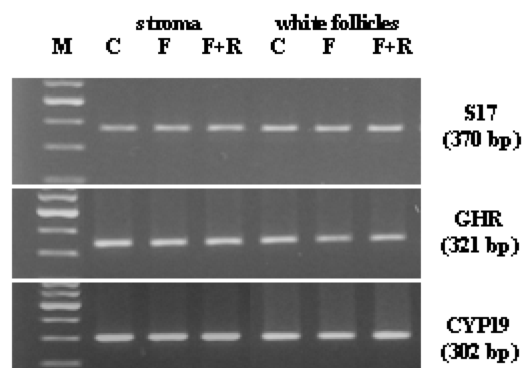

Fig. 1. RT-PCR analyses of GHR and aromatase gene expressions in the stroma and white follicles of the immature chicken ovary after fasting and re-feeding. C – control (fed *ad libitum*); F- fasted for 24h; F+R – fasted for 24h+re-fed for 24h; M – molecular weight marker (100 bp DNAladder). The data shown are representative of five birds.

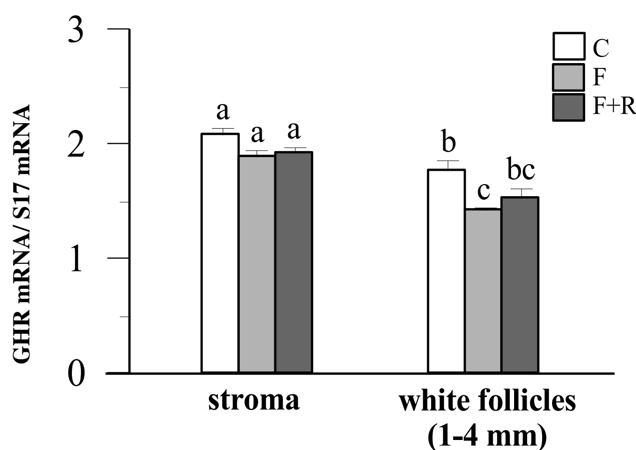

Fig. 2. Relative expression of GHR mRNA in the stroma and white follicles of the immature chicken ovary after fasting and re-feeding. C – control (fed *ad libitum*); F- fasted for 24h; F+R – fasted for 24h+re-fed for 24h. Each value represents the mean ± SEM of 5 determinations of the relative density of RT-PCR products compared with ribosomal protein S17. The means marked with different superscript letters are significantly different from each other (p<0.05).

DISCUSSION

In the present study the expression of GHR mRNA was found in all compartments of the immature chicken ovary, i.e. in the stroma and white follicles after short-term starvation and re-feeding. These re-

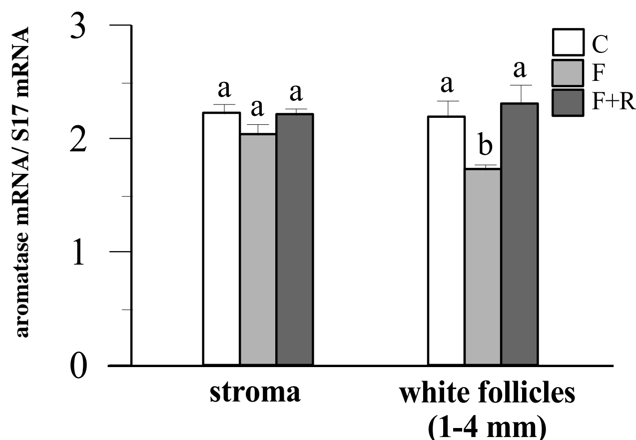

Fig. 3. Relative expression of aromatase mRNA in the stroma and white follicles of the immature chicken ovary after fasting and re-feeding. C – control (fed *ad libitum*); F- fasted for 24h; F+R – fasted for 24h+re-fed for 24h. Each value represents the mean ± SEM of 5 determinations of the relative density of RT-PCR products compared with ribosomal protein S17. The means marked with different superscript letters are significantly different from each other ($p<0.05$).

sults confirm and extend previous observations in growing hens (Heck et al., 2003; Hrabia et al., 2008). Considering the examined gen, the slighty higher level of GHR mRNA observed in the ovarian stroma, as compared with white follicles, may indicate greater responsibility of primordial follicles present in the stroma for GH. After 24 h of fasting a significant decrease in GHR mRNA expression in white follicles was noticed. The chicken ovarian white follicles are thus a site where GHR is downregulated in a situation of feed deprivation as it was previously observed in the chicken liver (Decuypere and Buyse, 2005). These findings strongly support the suggested participation of GH in ovarian functions in birds.

As the chicken ovary is a target tissue for GH action, as well as a place where sex steroids are produced and act, it represents a potential site of regulatory interaction between GH and steroids. Thus, to find any relationship between GH action and estrogen synthesis in the chicken ovary following fasting, in the present investigation the expression of aromatase mRNA, the rate-limiting enzyme of estradiol synthesis, was examined. It was found that the relative expression of *CYP19* gene in the stroma was on the same level as that in white follicles. It indicates that the ovarian stroma and white prehierarchical follicles are important sources of ovarian estrogens in birds. Short-term starvation caused a significant decrease in aromatase mRNA expression in white follicles. Interestingly, after 24-hour re-feeding, a significant increase in aromatase mRNA expression in white follicles, as compared with fasted hens, was observed. These changes indicate that feeding factors are of significance for regulation of aromatase mRNA expression and estrogen production. The author's present observations are in accordance, at least in part, with those previously reported and concerning the chicken ovary during a pause in egg laying induced by fasting. Namely, Proszkowiec-Węglarz et al. (2005) showed immunocytochemically a slight decrease in the activity of aromatase in the stroma and prehierarchical follicles after two days of fasting followed by a sharp increase during the next six days (including three days of fasting and three days of feeding every second day).

In the present study the important finding was the simultaneous decrease in both GHR mRNA expression and aromatase mRNA expression after feed withdrawal, followed by an increase in the expression of these genes after re-feeding. It may suggest an involvement of GH in the regulation of aromatase mRNA expression and finally estradiol synthesis. To the author's knowledge there are no data indicating any effect of GH on aromatase activity in ovarian follicles in avian species; however, a stimulating action of GH on aromatase activity has been found, for example, in the ovary of women (Mason et al., 1990; Tapanainen et al., 1992), pigs (Rak and Gregoraszczuk, 2008) and sea trout (Singh and Thomas, 1993). On the other hand, a decrease in estradiol synthesis may affect GHR expression and GH action within the ovary. From studies in mammalian models it is known that sex steroids modulate GH action at a number of levels, centrally at the hypothalamus and the pituitary by regulating secretion, and peripherally by modifying GH responsiveness. Estrogens attenuate GH action by inhibiting GH-regulated endocrine and metabolic functions of the human liver (for review see Meinhardt and Ho, 2006). GHR expression in tissues is also estrogen-regulated and appears to be tissue-dependent. Estrogens increase the GHR mRNA level in osteoblasts but not in the uterus. Moreover, estrogen decreased the GHR mRNA level in the rat brain, whereas ovariectomy has the opposite effect. At the cellular level, estrogens inhibit GH signalling in a concentration-dependent manner by inducing the expression of SOCS (suppressors of cytokine

signalling) proteins which are negative regulators of GH signalling (for review see LEUNG et al., 2004).

In conclusion, the results obtained indicate that short-term fasting affects GHR and aromatase mRNA expressions in white follicles of immature chickens. Simultaneous changes in the mRNA levels of the examined genes may suggest an involvement of GH in the regulation of aromatase gene expression in the developing ovary of chickens. On the other hand, a decrease in estradiol synthesis may affect GHR expression and GH action within the ovary.

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