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Effect of Short-Term Fasting on Caspase and Estrogen Receptor Gene Expression in the Chicken Ovary

Anna Hrabia[1] and Ewelina Jankoś

Department of Animal Physiology and Endocrinology, Agricultural University of Cracow, Al. Mickiewicza 24/28, 30-059 Cracow, Poland

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To evaluate the relationship between estrogens and occurrence of apoptosis in the chicken ovary during food intake disturbance, the expression of the initiator caspases-1 and -2 and executive caspase-3, as well as the estrogen receptor alfa (ERα) and beta (ERβ) mRNAs, in the ovary of sexually immature chicken has been examined after a short-term feed withdrawal. 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 examined gene expression was determined in the ovarian tissues by semi-quantitative RT-PCR. It was found that: (1) caspases-1, -2 and -3 and ERα and ERβ mRNAs were present in the ovary of immature chickens, (2) the relative level of the examined genes depended on the ovarian compartment, (3) the fasting caused a significant increase in ERβ mRNA expression in the white follicles, and (4) the re-feeding caused an increase in stromal ERα and caspase-2 expression and a decrease in ERα expression in the white follicles. The results obtained did not provide strong evidence for the interaction between estrogens and the activity of selected caspases in the ovary of growing chickens after 24-h fasting and re-feeding. It seems that short-term fasting does not have a significant impact on further functions of the chicken ovary. This finding is of special importance for the economics of commercial egg production.

Key words: caspases, ERs, fasting, ovary, chicken

INTRODUCTION

The number of eggs poduced by female domestic birds depends on the rate of differentiation, selection and atresia of ovarian follicles during their growth and maturation (Waddington et al., 1985). It is well known that the feeding level and metabolism rate influence the time of sexual maturation and reproductive activity. Numerous studies showed that food and water withdrawal leads to regression of the ovary and cessation of egg laying (Tanabe et al., 1981; Etches et al., 1984; Proszkowiec and Rząsa, 2001) and this phenomenon is used in commercial practice to induce moulting. In birds, small prehierarchical ovarian follicles are highly susceptible to atresia, whereas large, hierarchical follicles under normal physiological conditions are resistant to atresia (for review, see Johnson, 1996, 2000).

[1] rzhrabia@cyf-kr.edu.pl

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Follicular atresia is achieved by apoptosis triggered by a variety of intracellular and extracellular signals (Johnson and Bridgham, 2002). Sensitivity or resistance to apoptosis correlate with the expression of factors which are stimulators or supressors of this process. The molecular events participating in apoptosis are largely mediated by specific cysteine proteases - caspases (for review, see Johnson, 1996, 2002). The caspase family includes initiator caspases (-1, -2, -8, -9, -10) which can auto-catalyze to form the active subunits and executioner caspases (-3, -6, -7) which require other proteases, such as the initiator caspases, for processing. It has been found that caspases-1, -2 and -3 are involved in regression of the chicken ovary during moulting induced by long-term feed withdrawal (Anish et al., 2008), as well as regression of chicken postovulatory follicles (Sundaresan et al., 2008).

A large body of evidence indicates that estrogens demonstrate a protective effect against apoptosis in many different tissues including the reproductive ones. In chickens the circulating as well as the ovarian estrogen levels decrease during fasting, leading to the regression of the ovary (Proszkowiec and Rząsa, 2001). The biological, multiple effects of estrogens are mediated via two forms of estrogen receptors, alpha (ERα) and beta (ERβ), which are transcription factors and members of the intracellular receptor family (Segars and Driggers, 2002). Both forms of ER are present in the chicken ovary indicating the local role of estrogens in the ovary (Hrabia et al., 2008). The tissue-protective effect of estrogens consisting in the regulation of caspase gene expression and activity was revealed in the chicken oviduct (Monroe et al., 2002). The estrogen-dependent regulation of caspase activity in the chicken ovary is, however, unknown. Therefore, in the present study the effect of short-term feed withdrawal on the expression of initiator caspases-1 and -2 and effector caspase-3, as well as the ERα and ERβ mRNAs in the ovary of sexually immature chicken has been examined.

MATERIALS AND METHODS

Animals and Chemicals

The experiment was performed on 15-week-old Hy-Line Brown hens (layer strain; n=15) caged individually under a photoperiod of 14L:10D. The birds were divided into 3 equal groups: (i) fed *ad libitum*, (ii) fasted for 24h and (iii) fasted for 24h+refed for 24h. After the indicated time the chickens were decapitated; the ovaries were isolated, placed on ice 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 total RNA extraction.

The chemicals were purchased from the following companies: TRI-reagent (MRC, Inc., Cincinnati, OH, USA), RevertAid M-MuLV Reverse Transcriptase, Ribonuclease inhibitor, dNTP mix, $MgCl_2$, Pol Taq DNA Polymerase, buffers, molecular weight marker – 100 bp DNAladder (Fermentas, Vilnius, Lithuania), primers, oligo-dT_{18} (IBB, Warszawa, Poland). Other reagents were obtained from ICN Biomedicals (Aurora, IL, USA) or Sigma (St. Louis, MO, USA).

RNA isolation and RT-PCR analysis

Total RNA in ovarian tissues was extracted with TRI-reagent according to the manufacturer's recommendations. Two µg of total RNAs of each tissue were reverse-transcribed with RevertAid M-MuLV reverse transcriptase (200 U) and oligo-dT_{18} primers. The obtained cDNAs were used for PCR amplifications carried out according to Monroe et al. (2002) for caspases, Hrabia et al. (2008) for ERs and Hrabia et al. (2004) 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% Nonidet P40), 0.312 unit pol Taq DNA polymerase, 0.2 µmol sense and antisense primers, 0.2 mmol each dNTP, 1.5 mmol $MgCl_2$, and water. The applied amplification profiles and the primers for caspases, ERs and S17 are described in Table 1. 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. Expression levels of the examined genes mRNA were normalized with ribosomal protein S17 mRNA. The average abundance of five determinations was used for statistical analysis.

TABLE 1. Characteristics of primers and PCR conditions used in this study.

Gene	GenBank	Primer sequence	PCR product	PCR conditions
Caspase-1	AF031351.1	F: 5'-GATACGTGACTCCATCGACCC-3' R: 5'-CTTCTTCAGCATTGTAGTCC-3'	313 bp	95°C 30s, 55°C 60s, 72°C 2 min, 27 cycles
Caspase-2	NM_001167701.1	F: 5'-GTGGATTCCTGTATTGTAGC-3' R: 5'-ATCCACGCTTGGTGTTGC-3'	351 bp	95°C 30s, 55°C 60s, 72°C 2 min, 27 cycles
Caspase-3	AF083029	F: 5'-AGCAAGCGAAGCAGTTTTGT-3' R: 5'-TGCGTTCCTCCAGGAGTAGT-3'	300 bp	94°C 30s, 62°C 60s, 72°C 60s, 30 cycles
ERα	X03805	F: 5'-GTGCCTTAAGTCCATCATCCT-3' R: 5'-GCGTCCAGCATCTCCAGTAAG-3'	300 bp	95°C 30s, 58°C 30s, 72°C 30s, 30 cycles
ERβ	AB036415	F: 5'-TGATATGCTCCTGGCCATGAC-3' R: 5'-CTTCATGCTCAGCAGATGCTC-3'	304 bp	94°C 30s, 55°C 30s, 72°C 30s, 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

Statistical analysis

The data were analyzed by two-way ANOVA 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 chickens.

RESULTS

The expression of all the examined caspases mRNA and ERs mRNA was found in the analyzed ovarian tissues of growing chickens. As an amplification product there was a band with the expected size of 313 bp for caspase-1, 351 bp for caspase-2, 300 bp for caspase-3, 300 bp for ERα, 304 for ERβ and 370 bp for S17 (Fig. 1).

The highest relative expression was found for caspase-3, and the lowest – for caspase-2 (Fig. 2). In the stroma of the control, *ad libitum* fed hens, the expression of caspases-1, -2 and -3 was 1.17 ± 0.056, 0.99 ± 0.049 and 1.72 ± 0.033, respectively, and in the white follicles 1.02 ± 0.042, 0.16 ± 0.027 and 1.34 ± 0.078, respectively. There was no difference in the relative expression of caspase-1 mRNA between the stroma and the white follicles, whereas the expression of caspases-2 and -3 was higher in the stroma than in the follicles (507% and 28%, respectively, in the control group). There was not significant effect of short-term fasting on the expression of examined caspases mRNA in the chicken ovary. In the re-fed chickens the expression of caspase-2 mRNA in the stroma significantly increased, by 40%, as compared with the fasted group, whereas in the white follicles the expression tended to decrease, however, statistically not significantly in comparison with the fasted and control hens (Fig. 2).

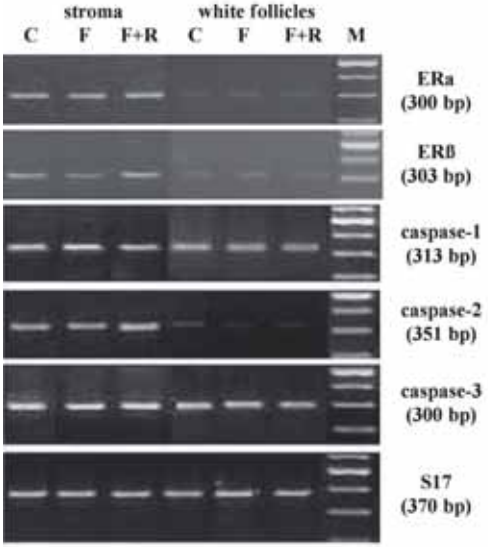

Fig. 1. RT-PCR analysis of caspase-1, -2 and -3 and estrogen receptor alpha (ERα) and beta (ERβ) gene expression in the stroma and white follicles of immature chicken ovary after fasting and re-feeding. C - control (fed *ad libitum*); F- fasted for 24h; F+R - fasted for 24h+re-fed for 24h; M - molecular weight marker (100 bp DNAladder). The data shown are representative of five birds.

The relative expressions of ERα and ERβ mRNAs in the ovarian stroma were similar. In the control chickens the levels of expression of ERα and ERβ mRNAs were 0.84 ± 0.085 and 1.06 ± 0.067, respectively. In the white follicles of control group the relative expression of ERβ mRNA, i.e. 0.21 ± 0.034 vs 0.11 ± 0.027 (Fig. 3). The expression of both the receptors was markedly higher in the ovarian stroma than in the white follicles (298% and 898%, respectively). The short-term fasting did not change significantly the expression of ERs mRNA in the stroma, although the expression of the receptors tended to be lower as compared with the control chickens. In the white follicles the expression of ERα mRNA was not affected by fasting, whereas the expression of ERβ mRNA increased by 102%. In re-fed chickens the expression of ERα mRNA in the stroma increased by 67% as compared with the fasted chickens. In white follicles of the re-fed group the expression of ERα mRNA significantly decreased as compared with the control and fasted chickens, by 40.1% and 42%, respectively. In the case of ERβ mRNA, the increased expression during fasting decreased to the control level (Fig. 3).

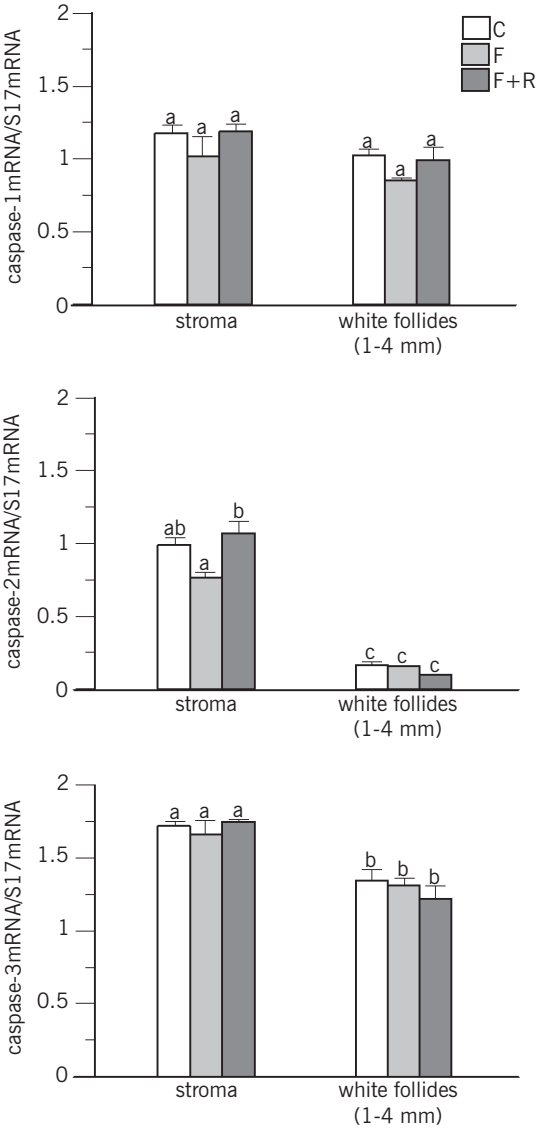

Fig. 2. Relative expression of caspase-1, -2 and -3 mRNAs in the stroma and white follicles of immature chicken ovary after fasting and re-feeding. C - control (fed *ad libitum*); F- fasted for 24h; F+R - fasted for 24h+re-fed for 24h. Each value represents the mean ± SEM of 5 determinations that were measured as relative density of RT-PCR products compared with ribosomal protein S17. Means marked with different superscript letters are significantly different from each other ($p<0.05$).

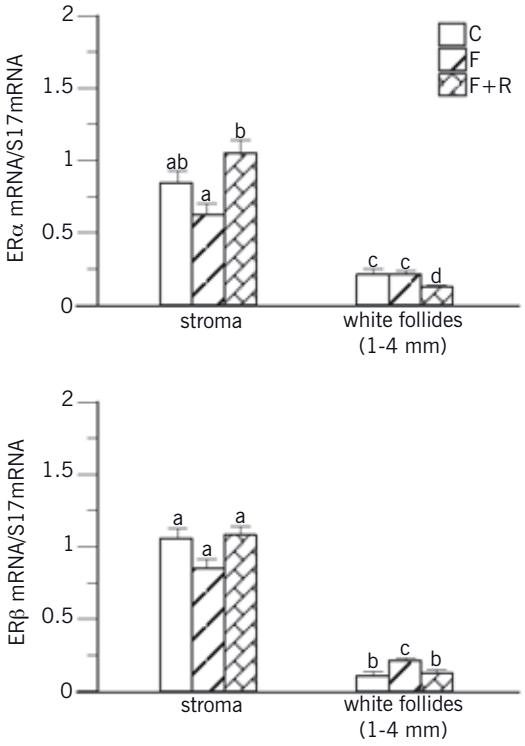

Fig. 3. Relative expression of ERα and ERβ mRNAs in the stroma and white follicles of immature chicken ovary after fasting and re-feeding. C - control (fed *ad libitum*); F- fasted for 24h; F+R - fasted for 24h+re-fed for 24h. Each value represents the mean ± SEM of 5 determinations that were measured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

DISCUSSION

In the present study the expression of initiator caspases-1 and -2 mRNAs and executor caspase-3 mRNA was found in all compartments of the immature chicken ovary after short-term starvation and re-feeding. These results confirm and extend previous observations in laying hens (Johnson and Bridgham, 2000; Anish et al., 2008). Considering the examined caspases, the highest relative expression was found for caspase-3 mRNA, and the lowest – for caspase-2. The highest level of caspase-3 mRNA in the ovarian compartments may results from the fact that this caspase joins both external and internal pathways of apoptosis. However, it should be noted that mRNA expression of caspase-3 does not reflect the real potential of a cell for apoptosis because this process requires the activation of caspase-3 by initiator caspases.

The next finding of the present study was that the relative expression of caspase-1 mRNA was similar in the stroma and white follicles, whereas the expression of caspases-2 and -3 mRNAs was significantly higher in the stroma than in the white follicles. The higher expression of these caspases in the ovarian stroma may suggest that primordial follicles, which are numerously present in the stroma, are more sentitive to apoptosis than white, prehierarchical follicles and that especially caspase-2 is involved in the initiation of cell death. Similarly, it was previously observed in the mammalian ovary that the kind of caspase inducing apoptosis depends on the cell type. In the oogonia, primordial follicles and preantral follicles this function is performed by caspase-2, whereas caspase-3 is responsible for apoptosis in the antral and preovulatory follicles (Johnson and Bridgham, 2002). The caspase-2 knock-out mice contain excess germ cells in the fetal ovary which suggests that caspase-2 is a key factor for normal atresia of primordial follicles (Bergeron et al., 1998). Sundaresan et al (2008) and Anish et al. (2008) also suggest the essential role of caspases-1 and -2 in initiation of apoptosis in the regression of chicken postovulatory follicles, as it happens during corpus luteum luteolysis in mammals (Rueda et al., 1997).

The results of the present investigation have shown that 24h of fasting does not change significantly the expression of all the examined caspases in the ovarian compartments. Contrary to the authors' present observations, in the hens being near the end of the production period, Anish et al. (2008) showed an increase in expression of caspases-1 and -2 in the non-compartmented ovary just after one day of starvation which was elevated during next several days. It is possible that the sensitivity of the ovary to apoptosis depends on the age of birds. Interestingly, after 24h of re-feeding, a slight, but significant increase in caspase-2 mRNA expression in the stroma, as compared with fasted hens, was observed. These changes might indicate that the feeding factors are of significance for regulation of initiator caspase-2 mRNA expression and induction of apoptosis and, consequently, the number of developing follicles in the ovary.

Monroe et al. (2002) observed that in the chicken oviduct the fast activation of caspases-1 and -2 on the transcriptional and translational level is caused by estrogen withdrawal. The lack of changes in mRNA expression of the examined caspases after 24h of starvation in the current investigation may be related to the lack of changes in the estrogen level. Namely, Proszkowiec and Rząsa (2001) found that in chicken a significant decrease in blood concentration of estradiol takes place on the third day of feed withdrawal.

To find any relationship between caspase expression and estrogen action in the chicken ovary following fasting, next, in the current study, the expression of both forms of ER was examined. The fasting did not change significantly the level of ERα mRNA in all compartments of the ovary and the expression of ERβ mRNA in the stroma. In the white follicles the level of ERβ mRNA was elevated. In chickens, prehierarchical follicles are steroidogenically active and ERβ is thought to be a mediator of estrogens in regulation of local steroidogenesis (Britt and Findlay, 2002). Thus, it is possible that the follicular response to food withdrawal is connected with ERβ gene expression and in consequence steroid production. After re-feeding a significant increase in ERα mRNA expression in the stroma and a decrease in the white follicles was noticed. This finding might suggest that estrogens by ERα which are thought to be involved in regulation of cell proliferation (Britt and Findlay, 2002) stimulate some primordial follicles to proliferate even if in other follicles atresia has been induced as indicates the profile of caspase-2 mRNA expression.

In conclusion, the current results showing only slight changes in the expression of selected caspa-

ses and ERs genes have not provided strong evidences for an interaction between the estrogens and the activity of caspases in the ovary of growing chickens after 24h of fasting and re-feeding. It seems that short-term fasting does not have a significant impact on further functions of the chicken ovary. This finding is especially important for the economics of commercial egg production.

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