Theriogenology 105 (2018) 126–134

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Alterations in apoptotic markers and egg-specific protein gene expression in the chicken oviduct during pause in laying induced by tamoxifen

Joanna K. Socha, Anna Hrabia*

Department of Animal Physiology and Endocrinology, University of Agriculture in Krakow, Al. Mickiewicza 24/28, 30-059 Krakow, Poland

ARTICLE INFO

Article history:
Received 12 July 2017
Received in revised form
14 September 2017
Accepted 17 September 2017
Available online 21 September 2017

Keywords:
Tamoxifen
Apoptosis
Egg proteins
Oviduct
Chicken

ABSTRACT

The aim of this study was to examine the cell apoptosis, gene expression and activity of caspases 2, 3, 8 and 9, and the mRNA expression of selected egg-specific proteins in the chicken oviduct during pause in egg laying induced by tamoxifen (TMX) treatment. The experiment was carried out on Hy-Line Brown laying hens. The control birds were treated subcutaneously with vehicle (ethanol) and the experimental ones with TMX at a dose of 6 mg/kg of body weight. Hens were treated daily until a pause in egg laying occurred and sacrificed on Day 7 of the experiment. Within the oviductal wall, the highest number of apoptotic cells (TUNEL-positive) was found in the luminal epithelium and the lowest in the stroma. The administration of TMX increased the percentage of apoptotic cells in the magnum, isthmus, and shell gland as well as immunoreactivity for caspases 3 and 9. Real-time PCR analysis revealed the segment-dependent mRNA expression of *caspases 2*, *3*, *8* and *9*. Treatment of hens with TMX elevated the level of *caspase-2* transcript in the infundibulum, *caspases 2*, *3* and *8* in the isthmus, and *caspase-9* in the shell gland ($P < 0.05$ - $P < 0.001$). As shown by fluorometric method TMX caused an increase in the activity of caspases 3 and 8 in the magnum, isthmus and shell gland, and the activity of caspases 2 and 9 in the isthmus and shell gland ($P < 0.05$ - $P < 0.01$). The expression of *ovalbumin*, *avidin* and *ovocleidin-116* mRNAs was decreased ($P < 0.05$ - $P < 0.001$), *ovocalyxin-36* mRNA level tended to increase, and *ovocalyxin-32* expression was not affected by TMX. The results obtained indicate that caspases are involved in the chicken oviduct regression during a pause in laying induced by TMX, and estrogen is involved in the regulation of examined caspase expression and activity. The changes in mRNA transcript levels of some examined egg-specific proteins after TMX treatment suggest that there is a relationship between estrogen action and the expression of these genes.

1. Introduction

During a pause in egg laying, which occurs in the chicken after approximately one year of egg production, the oviduct undergoes intensive functional and morphological alterations. Such changes require extensive tissue remodeling, which occurs, at least in part, via apoptosis: programmed cell death [1,2]. The control of cell death is based on competing pro- and anti-apoptotic signals, such as proteins from the Bcl-2 family, an inhibitor of T-cell apoptosis, survivin, and the apoptosis-inducing factor FasL [3–5]. The molecular process engaged in apoptosis primarily involves caspases – specific cysteine proteases whose activation results in the cleavage of numerous cellular proteins [6]. Activation of one or more initiator caspases, such as caspases 1, 2, 8, 9 and 10, in turn activates effector caspases (caspases 3, 6 and 7) leading to apoptotic cell death [3,5].

In many types of tissues, including the chicken oviduct, one of the crucial players in the protection against cell apoptosis is estrogen. As a result of its deficiency, the oviduct of immature chicks undergoes regression via apoptotic mechanisms [7,8]. Similarly, apoptosis-mediated oviduct regression occurs in hens during molting [1], a phenomenon accompanied by low concentrations of steroid hormones in blood plasma [2,9–11] and oviductal tissues [11].

The majority of biological actions of estrogen are mediated by the alpha and beta estrogen receptors (ERα and ERβ), members of

* Corresponding author.
E-mail addresses: anna.hrabia@urk.edu.pl, annahrabia@hotmail.com, rzhrabia@cyf-kr.edu.pl (A. Hrabia).

https://doi.org/10.1016/j.theriogenology.2017.09.024
0093-691X/

the nuclear receptor family [12]. These receptors act mostly as transcription factors activated by ligands and bind directly at estrogen response elements to target genes or indirectly to the AP-1 or Sp1 transcription factors [13,14]. Evidence of rapid cytoplasmic responses to estrogens is also increasing [12,15]. Previous studies in birds have revealed that tamoxifen (TMX), a non-steroidal type I anti-estrogenic modulator, competitively blocks ERs, binds to ERs with high affinity, and limits the transcription of estrogen-induced genes [16]. The antagonistic effects of TMX on the functions of the hen reproductive system have also been shown. In the chicken ovary, TMX significantly modulated the process of steroidogenesis and blocked ovulation [17]. Both lowered progesterone concentrations in blood plasma [18] and maintained expression and activity of metalloproteinase system members in the oviduct resulted in oviduct regression [18].

The avian oviduct consists of five segments (the infundibulum, magnum, isthmus, shell gland and vagina) with specific morphology and specialization in the production of egg-specific proteins. The magnum secretes most of the egg white proteins, including ovalbumin, which constitutes more than 50% of the egg white [19], and *avidin*, a protein which protects the egg against bacterial infections [20]. The isthmus produces proteins of shell membranes, and the shell gland (uterus) is the segment where hard eggshell is formed.

Previous observations indicate that TMX treatment induces chicken oviduct regression [18], an event realized, at least in part, by apoptosis [1], and that estrogen is involved in the protection against cell apoptosis in this organ [7,8]. Therefore, the present study was aimed at examining the rate of cell apoptosis and the process-related caspase (caspases 2, 3, 8 and 9) gene expressions, as well as their protein activity and localization in the chicken oviduct during a pause in egg laying induced by TMX treatment. This investigative goal is reasonable, because the precise mechanism of estrogen action in apoptotic events is poorly understood. Moreover, it is also interesting to determine whether exposure to TMX may affect the secretory activity of the chicken oviduct. Therefore, the mRNA expression of selected proteins in the magnum and shell gland has also been evaluated. For examination in the magnum we have chosen *ovalbumin* and *avidin*. In the shell gland the following eggshell-matrix proteins have been investigated: *ovocalyxin-32*, which plays a role in eggshell calcification and eggshell crystal orientation [21–23]; *ovocalyxin-36*, which plays a key role in the mechanism of natural defense of the egg against pathogens [24,25]; and *ovocleidin-116*, the most abundant eggshell-matrix protein associated with egg shape, eggshell thickness, and elastic modulus [26,27].

2. Materials and methods

2.1. Chemicals

In-situ cell-death detection kit (POD—horseradish peroxidase) was from Roche Diagnostics (Mannheim, Germany). The chemicals for immunohistochemistry were obtained from the following companies: rabbit anti-caspase-3 and rabbit anti-caspase-9 antibodies from Abcam (Cambridge, UK), hematoxylin QS, biotinylated goat anti-rabbit antibody, normal goat serum, normal rabbit serum and Vectastain ABC kit from Vector Laboratories (Burlingame, USA). Bradford protein assay kits from Bio-Rad (Hercules, CA, USA). The chemicals for RT-PCR were purchased from the following companies: TRI-reagent from MRC Inc. (Cincinnati, OH, USA), High-Capacity cDNA Reverse Transcription Kit, TaqMan Gene Expression Master Mix, TaqMan Gene Expression Assays, and Eukaryotic 18S rRNA Endogenous Control from Applied Biosystems (Foster City, CA, USA). Caspase fluorometric assay kits and lysis buffer were from BioVision (Milpitas, CA, USA). All other reagents were obtained from ICN Biomedicals (Aurora, IL, USA), Sigma (St. Louis, MO, USA) or POCH (Gliwice, Poland).

2.2. Animals and experimental design

The animal experiment was conducted according to a research protocol approved by the I Local Animal Ethics Committee in Krakow, Poland (approval no. 1/2013). The experiment was carried out on laying Hy-Line Brown hens at the age of 25 weeks, weighing 1.86 ± 0.036 kg on average. Birds were housed under a 14L:10D lighting schedule and were provided with water and commercial food *ad libitum*. Hens were randomly divided into two groups: control (n = 6) and experimental (n = 6). The control birds were treated subcutaneously with vehicle (ethanol) and the experimental one with TMX at a dose of 6 mg/kg of body weight in a volume of 0.3 mL, as described recently by Leśniak-Walentyn and Hrabia [18]. Hens were treated daily until a pause in egg laying occurred. Chickens were killed on Day 7 of the experiment and four oviductal parts, infundibulum, magnum, isthmus and shell gland, were rapidly isolated and weighed. Control hens were sacrificed 2 h after oviposition. Tissue samples, collected from the midportion of each segment, were immediately frozen and kept at −80 °C until the determination of caspase activity or were placed into RNAlater and stored at −20 °C until total RNA isolation. The other tissue fragments were fixed in freshly prepared 10% (v:v) buffered formalin, processed and embedded in paraffin wax for the subsequent localization of apoptotic cells and caspases, as described previously [28].

2.3. Evaluation of apoptosis by TUNEL assay

In order to estimate the rate of apoptosis, deparaffinized and dehydrated sections (6 μm thick) of the oviductal parts were incubated with proteinase K (20 μg/mL) in 10 mM TRIS-HCL, pH 7.4 at 37 °C for 15 min and blocked with 5% (v/v) bovine serum albumin in TBST buffer (Tris buffer saline + 0.1% v/v Tween 20) for 20 min. Apoptotic cells were localized by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) method using the *in situ* cell death detection kit POD, according to the manufacturer's recommendations. This kit measures apoptosis-related DNA strand breaks by labeling free 3′-OH DNA ends with modified nucleotides in enzymatic reaction. Negative controls were incubated without TdT. To visualize immunoreaction products, sections were incubated for approximately 4 min with a mixture of diaminobenzidine (DAB) and H_2O_2, and counterstained with hematoxylin. Slides were examined under a Axio Scope. A1 light microscope with an Axiocam 503 colour camera and ZEN 2.3 pro software (Carl Zeiss, Germany). Sections were viewed using an objective with 40× magnification. Cells containing condensed or fragmented nuclear chromatin characteristic of apoptosis exhibited a brown nuclear stain. Apoptotic cell frequencies were analyzed by counting TUNEL-positive and TUNEL-negative cells on 10 random areas including the luminal epithelium and tubular glands of each magnum, isthmus and shell gland, and averaged for each bird. In total 1000 cells were counted per each section with a computerized image-analysis system (MultiScanBase v. 14.02, Computer Scanning System, Warsaw, Poland). The resulting value was shown as the number of TUNEL-positive cells per 100 cells counted. The mean value for each oviductal tissue was calculated from six chickens. The infundibulum was excluded from apoptotic cell counting because of a lack of tubular glands in this oviduct section.