Domestic Animal Endocrinology xxx (2015) 1–9

Contents lists available at ScienceDirect

Domestic Animal Endocrinology

journal homepage: www.domesticanimalendo.com

Estradiol concentration and the expression of estrogen receptors in the testes of the domestic goose (*Anser anser f. domestica*) during the annual reproductive cycle

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ARTICLE INFO

Article history:
Received 25 July 2014
Received in revised form 7 November 2014
Accepted 2 December 2014

Keywords:
Estradiol
Estrogen receptor alpha
Estrogen receptor beta
Seasonal expression
Testis
Male domestic goose

ABSTRACT

Seasonal fluctuations in the activity of bird testes are regulated by a complex mechanism where androgens play a key role. Until recently, the role played by estrogens in males has been significantly underestimated. However, there is growing evidence that the proper functioning of the testes is associated with optimal estradiol (E_2) concentration in both the plasma and testes of many mammalian species. Estrogens are gradually emerging as very important players in hormonal regulation of reproductive processes in male mammals. Despite the previously mentioned, it should be noted that estrogenic action is limited by the availability of specific receptors—estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Interestingly, there is a general scarcity of information concerning the estrogen responsive system in the testes of male birds, which is of particular interest in exploring the phenomenon of seasonality of reproduction. To address this question, we have investigated for the first time the simultaneous expression of testicular ERα and ERβ genes and proteins with the accompanying plasma and testicular E_2 concentrations during the annual reproductive cycle of male bird. The research model was the domestic goose (*Anser anser f. domestica*), a species whose annual reproductive cycle can be divided into 3 distinct phases characterized by changes in testicular activity. It has been revealed that the stable plasma E_2 profile did not correspond to changing intratesticular E_2 profile throughout the experiment. The expression of ERα and ERβ genes and proteins was detected in gander testes and it fluctuated on a seasonal basis with lower level in breeding and sexual reactivation stages and higher level during the nonbreeding stage. Our results demonstrated changes in testicular sensitivity to estrogens in male domestic goose during the annual reproductive cycle. The seasonal pattern of estrogen receptors (ERs) expression was analyzed against the hormonal background and a potential mechanism of ERs regulation in bird testes was proposed. The present study revealed seasonal variations in the estrogen responsive system, but further research is needed to fully explore the role of estrogens in the reproductive tract of male birds.

1. Introduction

Testes of birds undergo dynamic seasonal variations in their morphology and function. The fluctuations in testicular activity determine the transition between stages of the annual cycle in seasonal breeders [1–4]. In domestic goose, the morphology and physiology of testes reflects 3 distinct stages of goose annual reproductive cycle: the breeding season, the nonbreeding season, and the sexual reactivation phase [5,6].

The functions of bird testes are regulated at various levels with the substantial role of the hypothalamic-pituitary-testicular axis [7,8]. Steroids, which act within

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http://dx.doi.org/10.1016/j.domaniend.2014.12.002

testes and produce feedback in upper levels of the reproductive axis, are the key factors in the hormonal game controlling seasonal breeding [3]. Testes function is supported mainly by androgenic action, and testosterone (T) is regarded as the principal male sex hormone [9,10]. However, the evidence accumulated in the last decade suggests that estrogens, which are generally considered "female hormones," are also involved in the regulation of the male reproductive system [11]. Furthermore, testicular steroid balance seems to be essential for normal testicular function and reproduction [12,13].

The testes of vertebrates synthesize remarkable amounts of estrogens via irreversible conversion of androgens by the aromatase enzyme [14]. In male mammals, gonadal estradiol (E_2) concentration exceeds E_2 plasma concentration in females many fold, pointing to the importance of estrogenic action within testes [13]. Indeed, studies of mammalian species revealed that aromatization of androgens to estrogens is crucial for the maintenance of male fertility [11,14,15]. Estrogens modulate spermatogenesis influencing the division of spermatogonial stem cells as well as the proliferation, differentiation, survival, and apoptosis of germ cells [15,16]. Moreover, E_2 has been found to improve sperm capacitation, acrosome reaction, and fertilizing capacity [12]. However, the potential role of estrogens in the reproductive system of male birds is still poorly understood.

The action of estrogens is mediated by specific receptors, in particular estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) which act as transcription factors that alter the expression of estrogen-dependent genes [17,18]. The expression of ERs is of vital significance especially in the light of the recent data that both excessive and deficient estrogen concentrations can lead to structural and functional disruption in male reproduction [12,19]. The expression of ERα and ERβ messenger RNA (mRNA) [20–24] and proteins [25,26] was detected in bird testes. Interestingly, very little is known about the seasonal pattern of ER expression and E_2 concentration in avian testes what is of particular interest with a view to steroids' ability to modulate the expression of their cognate receptors.

The objective of the present study was to determine whether the estrogen responsive system in avian testes is subjected to seasonal fluctuations. The expression of ERα and ERβ genes and proteins as well as the concentrations of gonadal and circulating E_2 were investigated under the seasonal aspect. The research model was the domestic goose, the species which despite thousands of years of domestication demonstrates strict seasonality in reproduction [5,6].

2. Materials and methods

2.1. Experimental animals

The study was performed on 1-year-old male domestic White Koluda geese (*Anser anser f. domestica*) obtained from local breeding farm. Birds were housed under standard feeding and lighting program [5]. Tissues were collected during the 3 characteristic phases of geese annual reproductive cycle: the peak of the breeding season (March), the nonbreeding season (July), and the sexual reactivation phase (November). Ganders were weighted, immobilized, and blood samples for radioimmunoassay (RIA) were collected from the brachial vein. Next, animals were killed by cervical dislocation and exsanguinated. Testes for RIA as well as for total RNA and protein isolation were frozen directly after harvesting in liquid nitrogen and kept at −80 °C for further processing. Testes for immunohistochemistry were obtained from ganders anesthetized (i.v. sodium thiopental 100 mg/kg of BW) and perfused intracardially with 4% paraformaldehyde in 0.1 M PBS as previously described [5]. All experiments were conducted on the approval of the Local Ethics Committee.

2.2. Radioimmunoassay

The concentrations of E_2 in plasma and in testis homogenates (n = 10 in each stage of the annual reproductive cycle of ganders) were determined by RIA. The quality of the anti-E_2 antibody for RIA was previously determined [27]. Briefly, each sample of plasma and testicular homogenate (100 mg testicular tissue in 5 mL methanol) were extracted twice with ethyl ether, and the organic phase was evaporated [5]. Next, extracts were reconstituted in 100 μL of RIA buffer, and samples were subjected to the RIA procedure. The intra-assay coefficient of variation was 0.33% and 1.14% regarding plasma and testicular samples, respectively. The assay sensitivity was determined at 1 pg per tube. All analyses were performed in triplicates. The results of E_2 concentration in plasma were expressed as pg per mL, whereas in testicular homogenates were calculated as pg per whole testis weight.

2.3. Total RNA isolation and reverse transcription

Total RNA from testis samples (n = 6 in each stage of the annual reproductive cycle of ganders) was extracted using the Absolutely RNA Miniprep Kit (Stratagene, USA) including DNase treatment. RNA concentration and purity was quantified spectrophotometrically (NanoDrop ND-1000, NanoDrop Technologies Inc, USA), and RNA integrity was confirmed electrophoretically. Next, RNA of checked quality was reverse transcribed into complementary DNA (Omniscript RT Kit, Qiagen, USA) following the manufacturer's protocol including the use of 0.1 μM oligo $(dT)_{15}$ primer (Roche, Germany) and 0.1 μM random nanomer $(dNT)_9$ (GenPandora, Poland) as described previously [5].

2.4. Real-time polymerase chain reaction

Specific primers for domestic goose ERα and ERβ genes were designed by Primer Express software (Applied Biosystems, USA) to flank 2 exons of each ER gene of the chicken origin (*Gallus gallus domesticus*). Glyceraldehyde-3-phosphate dehydrogenease (GAPDH) mRNA level was stable across experimental conditions (preliminary data not shown) and was used to normalize data of each ER gene expression [5]. Primer pairs used for real-time polymerase chain reaction (PCR) are shown in Table 1. The real-time PCR was optimized to achieve optimal reaction efficiency and the value of linear correlation (R^2) more than 0.99.