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Research paper

Seasonal expression of P450c17 and 5α-reductase-2 in the scented gland of male muskrats (*Ondatra zibethicus*)

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ABSTRACT

Cytochrome P450 17A1 (P450c17) is the key enzyme required for the production of androgenic sex steroids by converting progestogens to androgens. 5α-reductases are enzymes that convert testosterone (T) to dihydrotestosterone (DHT), which has a greater affinity for androgen receptors (AR) and stronger action than T. Our previous studies revealed that the scented glands of male muskrats expressed AR during the breeding and nonbreeding seasons. To further seek evidence of the activities of androgens in scented glands, the expression patterns of P450c17 and 5α-reductase 2 were investigated in the scented glands of male muskrats during the breeding and nonbreeding seasons. The weight and size of scented glands in the breeding season were significantly higher than those of the nonbreeding season. Immunohistochemical data showed that P450c17 and 5α-reductase 2 were presented in the glandular cells and epithelial cells of scented glands in both the seasons. The protein and mRNA expression of P450c17 and 5α-reductase 2 were significantly higher in the scented gland during the breeding season than those during the nonbreeding season. In addition, the levels of DHT and T in the scented gland were remarkably higher during the breeding season. Taken together, these results suggested that the scented glands of male muskrats were capable of locally synthesizing T and DHT, and T and DHT might play an important role in the scented glandular function via an autocrine or paracrine manner.

1. Introduction

Cytochrome P450 17A1 (P450c17) is a monooxygenase as a member of the cytochrome P450 superfamily localizing in the endoplasmic reticulum or mitochondria. P450c17 plays a critical role in sex steroid hormone synthesis by converting progestogens to androgens (Fluck et al., 2003; Miller and Auchus, 2011). Besides traditional steroidogenic organs like adrenal glands and gonads, P450c17 was also found in other tissues, including brains, skins and adipose tissues, implying that they were potentially sources of local sex steroid hormone (Dharia et al., 2004; Puche et al., 2002; Schonemann et al., 2012; Slominski et al., 2013). There are three isoforms of 5α-reductases: the type 1 and 2 isoforms possess the capacity to convert testosterone (T) to dihydrotestosterone (DHT) in the cell nucleus or cytoplasm, while the type 3 participates in N-linked protein glycosylation, with little functional abilities to reduce steroid substrates (Cantagrel et al., 2010; Nacusi and Tindall, 2011). Moreover, 5α-reductase 1 has the low affinity for steroid substrates and a high expression in the liver, which was generally considered as a catabolic agent to convert T into DHT for further degradation. In contrast, 5α-reductase 2 was taken as an anabolic entity because of a high substrate affinity and a predominant expression in male reproductive tissues and accessory sex glands, playing an obviously crucial role in normal male sexual development, and its abnormal expression caused abnormal male external genitalia development, small prostate, benign prostatic hyperplasia and prostate cancer (Mendonca et al., 2016; Thigpen et al., 1993; Windahl et al., 2011; Zhu and Imperato-McGinley, 2009). And the type 3 isoform was related with intellectual dysfunction and cerebellar and ocular defects (Cantagrel et al., 2010). Therefore, instead of type 1 and 3 isoforms, 5α-reductase 2 mainly takes part in androgen synthesis and promotion and maintenance of development and morphology of male androgen target organs and tissues.

Abbreviations: AR, androgen receptor; B, the breeding season; CSD, core secretory duct; DHT, dihydrotestosterone; EC, epithelial cells; GC, glandular cells; IC, interstitial cells; NB, the nonbreeding season; P450c17, Cytochrome P450 17A1; T, testosterone.

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In certain local tissues, DHT could be obtained directly from serum or from T being mainly synthesized in the testis, internalized in target cells by passive diffusion and then converted into DHT via 5α-reductases. DHT, for its similar association rate and slower dissociation rate to the androgen acceptor (AR), is a more potent agonist of AR than its precursor, T (Nacusi and Tindall, 2011; Saartok et al., 1984). Both T and DHT are natural ligands of the AR and, via binding to the AR, modulate normal growth and development of androgen-dependent organs: T is responsible for differentiation of the Wolffian duct system into the epididymis, vas deferens and seminal vesicle (Welsh et al., 2009), and DHT, converted from T in target organs, regulates development of the prostate and male external genitalia like penis and scrotum (Imperatomcginley et al., 1974; Marchetti and Barth, 2013; Nacusi and Tindall, 2011). In certain tissues, such as the rat prostate, T converting massively to DHT via 5α-reductases was considered as a means to effectively uptake, accumulate and utilize serum T to maintain the prostatic growth (Wright et al., 1999). Therefore, DHT, similar to T, also plays a critical role in the differentiation and development of androgen target organs.

The muskrat (*Ondatra zibethicus*) is a kind of medium-sized, semiaquatic rodent natively living in Canada, the U.S. and some places of northern Mexico (Li et al., 2011). Muskrats are seasonal breeders with sexually active period stretching 8 months from March to October (breeding season), during which the two scented glands of the muskrat near its tail will inflate and produce a huge amount of aromatic substance that is used as expensive Chinese medicine (Chen, 2007). It has been suggested that the scented glands showed seasonality of AR expression levels, indicating the scented glands were the target organs of androgens (Lu et al., 2014). Therefore, in the present study we further explored the action mechanism of androgens in the scented glands by detecting the expression patterns of P450c17 and 5α-reductase 2, as well as the concentrations of T and DHT in the scented glands.

2. Materials and methods

2.1. Animals

Adult male muskrats were obtained in January (n = 6) and June (n = 6) in 2016 from Xinji Muskrat Breeding Farm, Hebei, China. The muskrats were kept following a pattern of one male and one female in one enclosure. All of these animals were treated in accordance with the National Animal Welfare Legislation guidelines. All experimental procedures were approved by the Animal Ethics Committee at the Experimental Center of Beijing Forestry University in accordance with the guidelines. Each pair of scented glandular tissues was obtained from the male muskrat. One side of the scented glands was immediately fixed overnight in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) in 0.05 M PBS, pH 7.4 for histological and immunohistochemical observations; the other side was immediately stored at −80 °C until it was used for Western blotting and RT-PCR.

2.2. Histology

The scented glandular samples were dehydrated in ethanol series and embedded in paraffin wax. Serial sections (5 μm) were mounted on slides coated with poly-L-lysine (Sigma). Some sections were stained with hematoxylin-eosin (HE) for observations of general histology. The number of glandular cell nuclei was assessed with the method described by Kirby et al. (2005). The area of core secretory ducts (CSD) was calculated by using Computer Aided Design software according to ratio scale measured by ocular micrometer, and assessed with GraphPad Prism 5 software.

2.3. Immunohistochemistry

The serial sections of the scent glandular tissues were incubated with 10% normal goat serum to reduce background staining caused by the second antibody. The sections were then incubated with primary polyclonal antibody (1:200 dilution) against P450c17 (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) and (1:150 dilution) against 5α-reductase 2 (EterLife Ltd., London, UK) overnight at 4 °C. The control sections were treated with normal rabbit IgG (Sigma) instead of the primary antibodies. The sections were then incubated with a second antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin, using a rabbit ExtrAvidin staining kit (Sigma), followed by visualizing with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 ml of 0.05 M Tris-HCl buffer, pH 7.6, plus 30 μl H_2O_2. Finally, the reacted sections were counterstained with haematoxylin solution.

2.4. Western blotting

The scented glandular tissues were weighed and diced into small pieces using a clean razor blade. The tissue was homogenized in a homogenizer containing 300 μl of 10 mg/ml PMSF stock and incubated on ice for 30 min while maintaining the temperature at 4 °C throughout all the procedures. Then, homogenates were centrifuged at 12,000×*g* for 10 min at 4 °C. Protein extracts (20 μg) were mixed with a quarter of volume of 4× Laemmli sample buffer. Equal amount of each sample was loaded and run on a 12% SDS-PAGE gel at 18 V/cm and transferred to polyvinylidene fluoride membranes using a wet transblotting apparatus (BioRad, Richmond, CA, USA). The membranes were blocked in 5% skimmed milk powder for 1 h at room temperature. Primary incubation of the membranes was carried out using a 1:150 dilution of anti-P450c17 antibody (Beijing Biosynthesis Biotechnology Co., Ltd.) and 1:200 of anti-5α-reductase 2 antibody (EterLife Ltd.) for 16 h at 4 °C. Secondary incubation of the membranes was carried out using a 1:1000 dilution of goat anti-rabbit IgG tagged with horseradish peroxidase for 60 min. Finally, the membrane was colored with 10 mg 3,3-diaminobenzidine (Wako) solution in 50 ml phosphate buffer (0.03 M) plus 3 μl H_2O_2. The intensities of the bands were quantified using Quantity One software (ver. 4.5, Bio-Rad Laboratories) and expression ratios were calculated.

2.5. RNA isolation

Total RNA from each sample was extracted using TRIzol Reagent (Invitrogen Co., CA, USA) according to the manufacturer's protocol. Approximately 0.1 g of scented glandular tissues was thawed and immediately homogenized in 1 ml of TRIzol reagent. The homogenate was incubated for 5 min at room temperature to allow the complete dissociation of nucleoprotein complexes. After the addition of 0.2 ml of chloroform, the mixture was vigorously shaken for 15 s at room temperature and centrifuged at 12,000×*g* for 15 min at 4 °C. The aqueous phase was then transferred to a fresh tube, and an equal volume of isopropanol was added. Then the sample was kept for 10 min at room temperature. RNA was precipitated by centrifugation at 12,000×*g* for 10 min at 4 °C. The RNA pellet was washed twice with 70% ethanol, briefly dried under air, and dissolved in 50 μl of diethylprocarbonate-treated water.

2.6. RT-PCR

The protocol for RT-PCR has been described previously in detail (Lu et al., 2014). Briefly, the first-strand cDNA from total RNA was synthesized using StarScript II Reverse Transcriptase and oligo (dT) 18 primer by TIANScript RT Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The amplification was performed