Hindawi Publishing Corporation
International Journal of Zoology
Volume 2014, Article ID 457830, 6 pages
http://dx.doi.org/10.1155/2014/457830

Research Article

The Involvement of the Androgen Receptor in the Secretion of the Epididymal *corpus* in the Lizard *Podarcis sicula*

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Received 25 September 2013; Accepted 9 December 2013; Published 29 January 2014

Academic Editor: Roger P. Croll

A crucial role in the maintenance of male reproductive functions is carried out by the androgen through its receptor in balance with the estrogen receptors (ERs). The distribution of the androgen receptor (AR) is well documented in the testis and in the reproductive tissues of mammals but the findings about the AR in nonmammalian vertebrates and in particular in reptiles are very scarce. Here by means of *in situ* hybridization (ISH) we investigated the AR expression along the epididymal channel (*efferent ductules*, *corpus*, and *cauda*) of *Podarcis sicula* during the mating and nonmating period. The results show that in this seasonal breeding species the AR expression pattern is always constant throughout the epididymis. The administration of estradiol-17β in the mating period does not affect the AR expression but inhibits the secretory activity of the epididymal *corpus*. To verify the expression pattern of ERs, we also conducted ISH investigations on adjacent sections with ERs probes. The findings suggest that AR induces the secretory activity in the epithelial cells of the epididymal *corpus* and confirm our previous results that showed the role of ERalpha (ERα) as switch off for the secretion of this compartment.

1. Introduction

The epididymis can be regionalized, depending on the species, into the initial segment or caput which includes the *efferent ductules*, the middle piece or *corpus* characterized by a high secretory activity, and terminal segment or *cauda*. In these compartments the maturation of spermatozoa takes place before ejaculation [1–4].

The majority of studies about the regulation of epididymal functions concern the expression of the steroid hormone receptors, that is, androgen (AR) and/or estrogen (ERα and ERβ) receptors in mammals [5–10].

In nonmammalian vertebrates, little is known about the expression of AR in the epididymis [11, 12].

In this paper the expression of the androgen receptor is detected in the lizard *Podarcis sicula*.

The histological structure of the *Podarcis* epididymis, the evolution of its reproductive cycle, and the pattern of steroid hormones have been already described in detail [13–15]. Briefly, in this lizard the epididymis is a channel of 7-8 mm in length and has been divided into three regions according to the morphological and functional characteristics of the surrounding epithelium [15]. In the region defined *caput* are including small *efferent ductules* with ciliate cubic epithelium that does not change during the year [15]. Conversely, notable changes are found in the epithelium of the middle piece defined *corpus*. This epithelium is constituted by cubic cells during the winter reproductive stasis when the lumen is empty. During the mating period the epithelium becomes cylindrical and the cells appear elongated, often, binucleated, and engaged in a massive secretory activity. In this period the lumen is filled with spermatozoa and secretory granules that pass in the third region, *cauda*, whose epithelium does not show any changes throughout the year. The lumen of the *cauda* is empty during the nonreproductive season [15]. In *Podarcis*, we recently found that the expression of ERα may act as a switch off for the secretory activity of the epididymal *corpus* [15].

Now by means of *in situ* hybridization (ISH) with the homologous *Podarcis* AR probe just obtained, it is demonstrated that in this lizard the AR expression is continuously present along the epididymis also in animals experimentally treated in the mating period with estradiol-17β (E_2). This expression may be related to the morphophysiology of the epididymal channel during the annual reproductive cycle. To compare the AR expression to the known ERs fluctuation [15] ISH with ER and ERβ probes on adjacent section was performed.

2. Material and Methods

2.1. Animals. Adult males of lizard *Podarcis sicula* (about 7.5–8 cm snout vent) were captured near Naples (Italy), for two consecutive years, during the mating period (spring-early summer) [$n = 8$] and winter stasis (fall-winter) [$n = 8$], kept in terraria at natural temperature and photoperiod and fed *ad libitum* with larvae of mealworm. Some samples ($n = 6$) from the mating period were experimentally treated intraperitoneally with estradiol-17β (168 ng/100 μL) in reptile physiological solution (NaCl 0.07%) every other day for 2 weeks [15]. In parallel, three males were intraperitoneally injected with physiological solution (100 μL) every other day for 2 weeks. The animals were killed by decapitation after anaesthesia in ice and the testes with the attached epididymis were immediately excised and processed for the planned analyses. All efforts were made to avoid animal suffering.

Authorization to capture the animals for experimental treatments was granted by the Italian Ministry of the Environment (Auth. SCN/2D/2000/9213).

2.2. Digoxigenin-Labeled Probes. cDNA for AR was obtained by RT-PCR from the total *Podarcis* testis RNA and amplified with forward primer 5′-TGGGCAACCTGAAGATGC-3′ and reverse primer 5′-ACCCCATGGCGAAAATCAT-3′ designed on the known AR sequences of some reptiles: *Trimeresurus flavoviridis* (AB548300.1), *Elaphe quadrivirgata* (AB548301.1), *Anolis carolinensis* (AF223224.2), *Calotes versicolor* (AF275370.2), *Trachemys scripta* (DQ848989.1), *Pseudemys nelsoni* (AB301061.1), *Cnemidophorus uniparens* (S79938.1), *Alligator mississippiensis* (AB186356.2), and *Leiolepis reeversii rubritaeniata* (AB490385.1).

First strand cDNA (3 μL) was used as template in PCR amplification (final volume 25 mL). The PCR thermal setting was as follows: 4 min at 94°C; 38 cycles of 30 s at 94°C; 40 s at 50°C; 1 min at 72°C; 7 min at 72°C. The obtained fragment was sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and run on the ABI PRISM 310 Genetic Analyzer and compared to GeneBank database.

The ERα and ERβ probes were obtained as already described [15, 16].

2.3. Histology and In Situ Hybridization (ISH). The epididymis excised from the testes was fixed in Bouin's fluid [17], dehydrated in alcohol increasing gradation, clarified in xylene, and embedded in paraffin. Sections 7 μm in thickness were obtained with Reichert-Jung 2030 microtome. Some sections were stained with Mallory's trichrome modified by Galgano [17].

The ISH was performed on adjacent sections with AR, ERα, or ERβ probes and was carried out as described in the liver and epididymis [15, 18]. Briefly, the dewaxed sections were treated with proteinase K (10 μg/mL) at 50°C for 10 min. Digoxigenin labelled probes were used at a concentration of 80 ng/100 μL in hybridization buffer [Tris-HCl 0.02 M pH 7,5; NaCl 0.3 M; EDTA 0.01 M; DTT 0.1 M; formamide 50%; Denhardt's 1X; tRNA 100 μg/mL; ss-DNA 100 μg/mL] overnight at 50°C in a moist chamber. The slides were incubated with RNasi mix at 37°C for 30 min and in the same mix without RNasi at 37°C for 30 min, washed in 2x SSC for 3 min, in 0.1x SSC at 60°C 15 min, and in NTP, and then incubated in 2% blocking solution [Roche Diagnostics, Mannheim, Germany] in maleic acid buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5] for 1 h. The sections were kept overnight at 4°C with an alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Diagnostics) (1 : 2500) in blocking solution and rinsed in NTP [Tris-HCl 0,1 M pH 7.5; NaCl 0.15 M] buffer for 30 min and in NTM buffer [Tris-HCl 100 mM pH 9.5, MgCl 50 mM, NaCl 100 mM] for 30 min. Finally, the sections were kept in the dark at room temperature in the colour detection substrate solution nitroblue tetrazolium and 5-bromo-4-chloro-3′-Indolylphosphate (BCIP/NBT) as recommended by manufacturer' (Roche) in NTM until appearance of the colour. To exclude a cross-link with genomic DNA some other adjacent sections were treated with DNAsi. Control sections were obtained by omitting incubation with the probes.

3. Results

3.1. Sequencing of Podarcis AR. The product of reverse-transcriptase-PCR from *Podarcis* testis gave a single band of about 300 bp in size (Figure 1(a)). BLAST analysis of the obtained nucleotide sequence (NCBI data bank) revealed high identity (83%–79%) to the AR sequences reported in several reptiles (Figures 1(b) and 1(c)). This partial sequence of *Podarcis* AR cDNA was annotated in EMBL Gen Bank (Accession number: JQ219668).

3.2. ISH with AR Probe. The homologous AR probe gave positive signals continuously throughout the mating period and winter stasis in the *efferent ductules*, in the *corpus* (Figures 2(a) and 2(c)), and in the *cauda* (one for all Figure 2(b)).

The epididymal tract of samples treated with estradiol 17-β (E_2) in the full mating period showed the expression of AR in the *efferent ductules* (data not shown), *corpus* (Figure 1(d)), and *cauda* (data not shown) as in the natural samples. Control animals treated with physiological solution showed the same expression already described for the untreated males (data not shown).

The sections incubated by omitting the AR probe gave always negative results (data not shown).

3.3. ISH with ERs Probe. The ISH results on ERs expression confirm what has already been found by us in the epididymis of *Podarcis* [15]. In the mating period the ERα expression did not occur in the secreting *corpus* (Figure 3(a)) but was

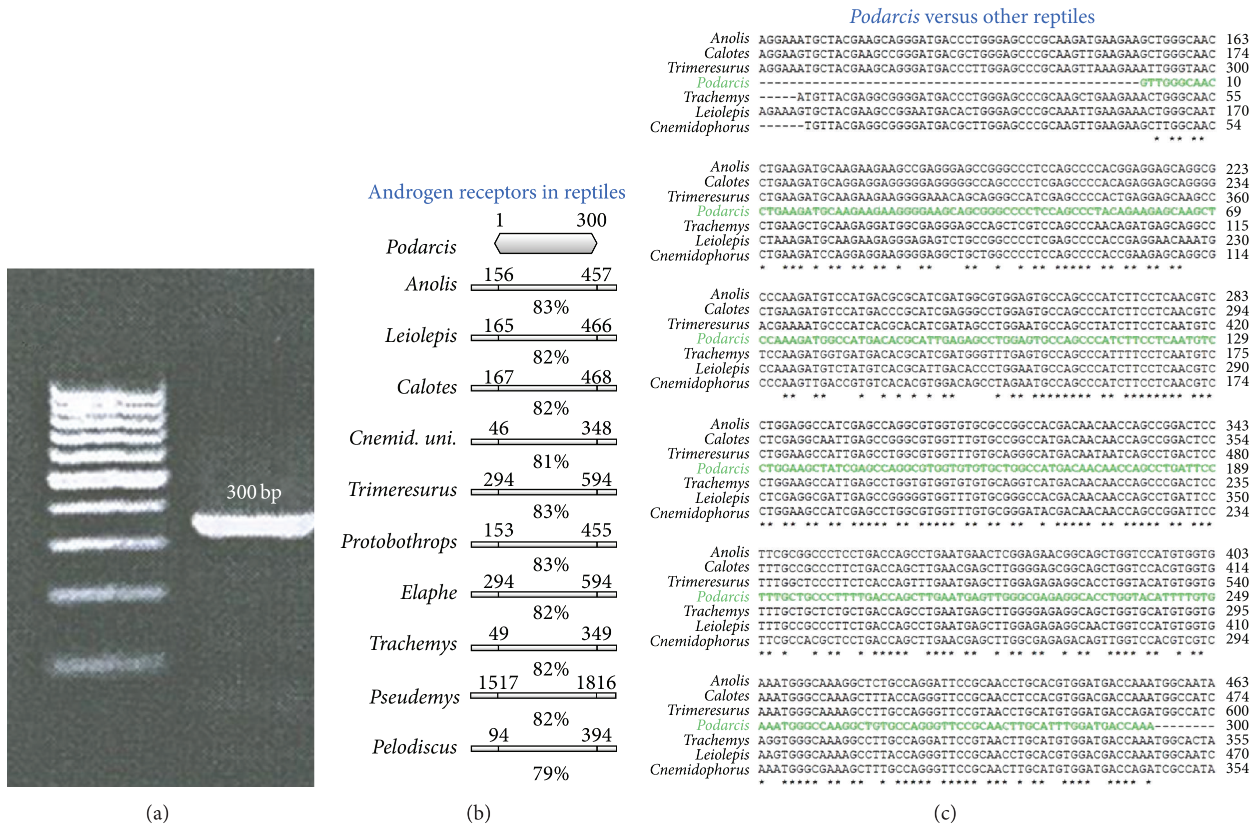


Figure 1: Sequencing of androgen receptor. (a) Agarose gek of RT-PCR on cDNA of *Podarcis testis.* (b) Similarity of predicted amino acid sequences of many reptiles with *Podarcis* AR. (c) BLAST alignment of the nucleotide sequence of AR in *Podarcis* compared to other reptiles as *Anolis*, *Calotes*, *Trimeresurus*, *Trachemys*, *Leiolepis*, and *Cnemidophorus*.

evident in the *efferent ductules* (Figure 3(a)) and *cauda* (data not shown). During the winter stasis the ERα expression remained evident in the *efferent ductules* (Figure 3(b)) and *cauda* (data not shown) and occurred in the *corpus* devoid of secretion in this period (Figure 3(b)).

The ERβ probe gave positive signals continuously throughout the mating period and winter stasis in all the epididymal compartments (data not shown).

In the animals treated in the mating period with E_2 an intense ERα expression took place in the *corpus* and the secretory activity resulted inhibited; the *efferent ductules* and *cauda* were still positive (data not shown). ERβ-mRNA, despite the E_2 treatment, was unchanged compared with untreated animals (Figure 3(c)).

4. Discussion

The epididymis of the lizard *Podarcis sicula* is characterized by a cyclic secretion that occurs in the *corpus* during the mating period when this compartment is characterized by a cylindrical epithelium producing a great amount of secretory granules released into the enlarged lumen where many spermatozoa are travelling from the *rete testis*. In the winter stasis the cuboidal epithelium surrounds a small, totally empty lumen [15]. Our previous results demonstrated that this cyclic secretory activity may be negatively regulated by the expression of ERα. In the lizards treated with E_2 during the mating period the epididymal structure becomes equivalent to that observed during the winter stasis: the epithelium is reduced in height and the secretory activity stops [15].

The present findings reveal that in *Podarcis* the epididymis may be also androgen dependent and the expression of AR is constant during the reproductive cycle and along the whole epididymal length, *efferent ductules*, *corpus*, and *cauda*.

The dependence of the epididymis on androgens is known [8] and in several mammalian species the presence of AR has been widely demonstrated by various methodological approaches [6, 19–25]. In mouse and rat the AR is expressed in a cell-type-specific manner and the protein expression is higher in the *caput* and *corpus* [9]. AR-mRNA and protein are also present in the human with a falling concentration from the *caput* to the *cauda* [8]. It is also known that the androgens can autoregulate the expression of AR-mRNA promoting AR protein stabilization too [26].

Among nonmammalian vertebrates the AR protein was found in the epithelial cells lining the *efferent ductules* and in the epididymal tubules of cockerel [27].

In rooster and drakes a different sensitivity of the epididymal segments to androgen has been suggested [11]. In reptiles immunoreactive AR was recorded only in the epididymis of green turtle *Chelonia mydas* [12].

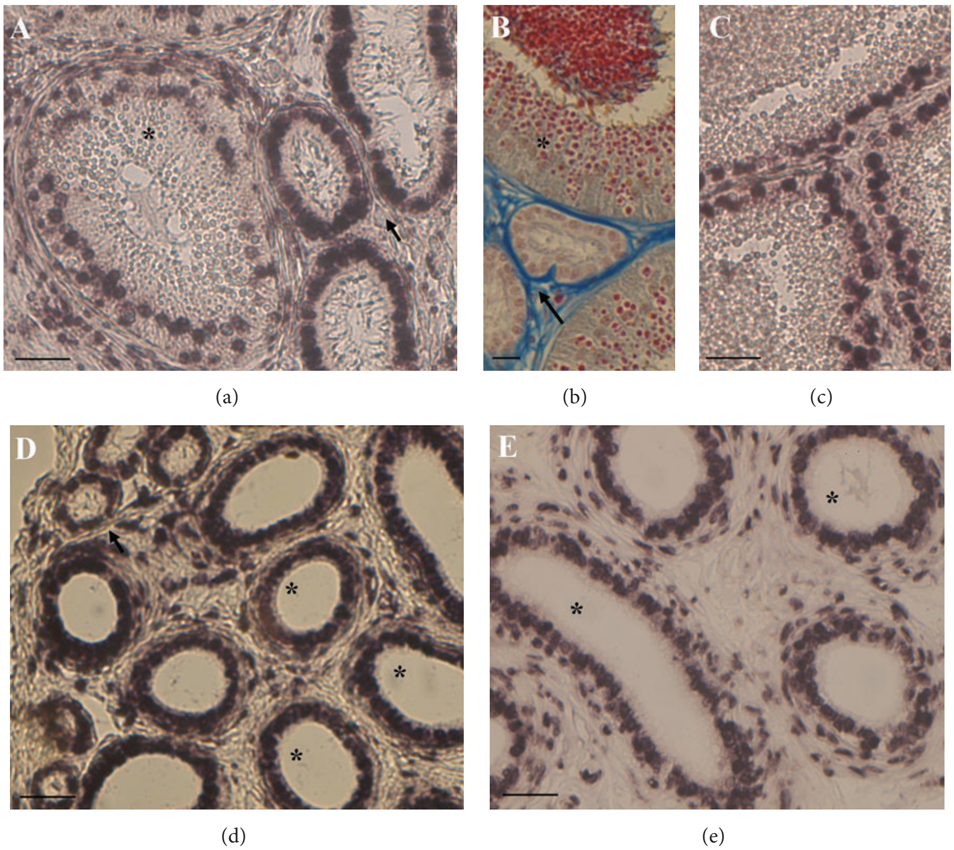
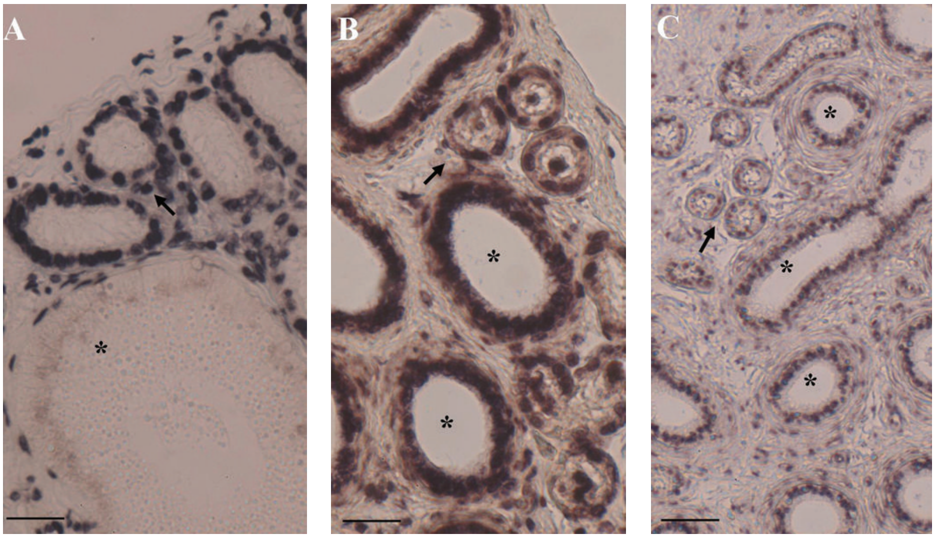


Figure 2: Changes in the morphology and unchanged expression of AR in the epididymal channel of *Podarcis* in the natural cycle or in samples treated with E_2 during the mating period. (a) Mating period: positive signal for AR-mRNA in the epithelium of the *efferent ductules* (↑) and *corpus* (∗). (b) Staining with Mallory's modified trichrome-mating period: note the ciliated cubic cells surrounding the efferent ductules (↑) and the elongated actively secreting cells of the *corpus*in which lumen is filled with secretory granules (red) and spermatozoa (dark blu). (c) Mating period: positivity for AR-mRNA in the *cauda*. (d) Winter stasis: both the *efferent ductules* (↑) and the *corpus* (∗) are still positive. (e) The nonsecretory epithelium of the *corpus* (∗) expresses AR-mRNA also in E_2 treated males in the mating period (∗). The bar is 30 μm.

In *Podarcis* it is now shown that the AR-mRNA is continuously expressed in the *efferent ductules*, *corpus*, and *cauda* regardless of the reproductive moment of this seasonal breeder. These results seem in contrast with the plasma levels of testosterone that reach the maximum during the mating period and falls down at the end [14]. Since the endotesticular profile of testosterone shows significant level at the end of mating remaining discrete throughout the reproductive cycle [14], it is possible that the constant presence of AR-mRNA may be related to the intratesticular level of androgen even if at different concentration.

The simultaneous presence of several receptors in the same cells raises questions about the receptors cross talk in the male reproductive system. The coexistence of the androgen and estrogen receptors (ERα and ERβ) in male gonad has been discussed in mammals [22, 24, 28, 29] and, among the nonmammalian vertebrates, only in the turtle [12]. In mouse the distribution of ERβ in the epididymis was similar to that of AR [28]. In rat ERβ is constitutively expressed in the *efferent ductules* while AR and ERα are selectively modulated by their own ligand [24]. Physical interactions between AR and ERα, resulting in the estradiol-induced modulation of AR transcriptional activity, have been described to indicate receptors interplay. These interactions arise between the C-terminal ERα ligand-binding domain and the N-terminal AR transactivational domain or with the full-length AR. ERβ did not interact with AR [30]. Furthermore, multiple consensus sequences in the hamster AR promoter region recognizes, as transcription factors, ER or AR itself [31].

We recently described in *Podarcis* that the expression of ERα stops during the matings when the cells of the *corpus* are involved in its secretory activity and occurs after E_2 treatment that inhibits the secretion. ERβ expression does not change neither after E_2 treatment [15]. The present observations, confirming these pathways for the ERs, show at same time the constant expression of AR throughout the reproductive cycle even in the animals treated with E_2. On the basis of these results it is possible to suggest that in *Podarcis* AR expression induces the secretion in the cells lining the *corpus* when the ERα expression does not occur. Conversely, during the winter stasis and in E_2 treated males when the secretory activity of the *corpus* is inhibited, AR and ERα are coexpressed.



(a) (b) (c)

FIGURE 3: ISH with Erα or ERβ homologous probes on the epididymis. (a) Mating period: Erα-mRNA is evident in the epithelium of the *efferent ductules* (↑); no expression in the secretory epithelium of the *corpus* (∗). Note in the corpus the enlarged lumen (∗). (b) Winter stasis: the Erα expression is evident in the *efferent ductules* (↑) and in nonsecreting *corpus* (∗). (c) E_2 treated males in mating period: ERβ-mRNA is expressed in the *corpus* (∗) and in the *efferent ductules* (↑). Note the restricted empty lumen of the *corpus* (∗). The bar is 30 μm.

5. Conclusions

In conclusion in *Podarcis* AR is expressed in a constant manner in the whole epididymal length throughout the reproductive annul cycle while the expression of ERα is cyclic in the *corpus*. In particular, it is possible to argue that the secretory activity of the epididymal *corpus* can be promoted by AR and inhibited by ERα. The role of ERβ whose expression does not change neither after E_2 treatment remains Unsolved. ERβ may be constitutively expressed and could ensure the activity of AR by modulating the expression of ERα.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The author is grateful to her tutor and teacher Professor Ermelinda Limatola for critical revision of the paper.

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