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Sequence analysis of retinoic acid receptor α, β and γ isoforms in the lizard, *Podarcis sicula*☆

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This paper is dedicated to the memory of Giuseppe Falcone, a dear friend prematurely died.

Abstract

Vitamin A and its principal biologically active derivative, retinoic acid (RA), play a fundamental role in diverse processes, such as proliferation, differentiation, morphogenesis, metabolism and apoptosis of many types of cells. In addition, RA has been shown to be involved in the regulation of testicular function. These effects are mediated by interaction with two families of nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR), each with three subtypes α, β and γ. The physiological involvement of retinoids in testicular function has been conducted mainly in mammals. Recently, we found that exogenous all-*trans*-retinoic acid impairs spermatogenesis and enhance testicular germ cell apoptosis in the lizard, *Podarcis sicula*, a seasonal breeder. To further investigate the role of retinoic acid in lizard, we focus this work principally on the characterization of lizard retinoic acid receptors (α, β and γ isoforms).

RARα is 2720 bp long with a putative ORF between 699 and 2133. A Kozac sequence is present at 696 and a putative poly-adenilation site is present in position 2612. The RARα sequence shares 87% homology with mouse RARα mRNA while it has 76 and 80% homology with lizard RARβ and γ mRNAs. RARβ is 2478 bp long showing a putative ORF between 196 and 1543. A canonical Kozac sequence is present at 193 and a putative poly-adenilation site is present at 2294. RARβ shares 91% homology with mouse RARβ mRNA and has 76% homology with both RARα and γ. RARγ is 2416 bp long. With a putative ORF between 444 and 1818. A Kozac sequence is present at 441 and a putative poly-adenilation site is present at 2288. RARγ shares 86% homology with mouse RARβ mRNA and has 80 and 76% homology with both RARα and β respectively. It is worth to note that, as in mouse, the 5′UTR of all isoforms is TATA and CAAT less. Both Northern blot and PCR analyses indicate that lizard testis expresses only RARα and RARβ mRNAs, while RARγ mRNA transcript was not found. In the period analysed, RARβ was expressed during the gonadal full activity (May) and RARα was present in the post-reproductive period (August). During the autumnal recrudescence (October) RARα and RARβ are co-expressed and, as indicated by quantitative PCR analysis, RARβ mRNA levels are lower than RARα ones. Thus, the appearance and abundance of each receptor correspond to a specific phase of lizard reproductive cycle, allow us to hypothesize that each RAR subtype could play a specific role in the regulation of spermatogenetic activity.

The results of the present study show, for the first time, the characterization of RAR mRNAs in the testis of lizard *P. sicula*, whose expression is related to the different phase of reproductive cycle. Moreover, the γ form, is principally expressed in the skin during the March–July period, having probably a role in regulating skin homeostasis and colour livery, which are important factor in mating during the reproductive cycle.

Keywords: Retinoic acid receptor mRNA; Expression; Lizard; Annual cycle

1. Introduction

Vitamin A (i.e. retinol) and its biologically active derivatives, that include all-*trans*-retinoic acid (atRA), dide-hydroretinoic acid (ddRA) and the 9-*cis*-retinoic acid isomer (9cRA) [1], together with a large repertoire of synthetic

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analogs, are collectively referred as retinoids. In contrast to most hormones, which are synthesized, processed and/or released in response to physiological cues, retinol is a nutrient that, under normal dietary conditions, is steadily present at relative high concentrations in plasma [2].

Since the 1920s, it has been known that retinol is essential for the normal production of spermatozoa [3–5]. In the testis of vitamin A-deficient (VAD) rats, spermatogenesis is arrested at early meiosis, and all of the advanced spermatocytes and haploid germ cells are degenerated [6–8]. The only germ cells that remain in the VAD testis are the stem cell spermatogonia, the type A_1 spermatogonia, and a few preleptotene spermatocytes from stage VIII in the cycle of the semiferous epithelium [7–9]. Spermatogenic function is restored if retinol is injected or replaced in the diet [6,7,10].

The effects of retinoids are though to result from interactions with specific nuclear receptors [11,12] that are members of the steroid-thyroid receptor superfamily and as such are considered to be ligand-dependent transcription factors [13,14].

Two families of nuclear retinoid receptor have been described from mouse to human, the retinoic acid receptors (RARs) [11,12,15,16] and the retinoid X receptors (RXRs) [17,18]; both families have been, also, identified in a variety of other species including chicken [19,20], newt [21,22] and frog [23,24]. The amino acid composition of RAR proteins is highly conserved (>95% and 85% identity in the DNA- and ligand-binding domain, respectively) between receptor homologous in different species [25]. RARs and RXRs consist of three receptor subtypes referred to RARα, -β and -γ or RXRα, -β and -γ, the transcription of which results in several isoforms [11,12,15,16,18,26,27].

A large number of studies have demonstrated heterodimerization of one RAR with one RXR [28–31], and it is suggested that the functional receptor unit is a heterodimer, that bind a specific RA response element (RARE) with high affinity, although, the RXRs are able to activate genes via homodimers [32]. Among the naturally occurring retinoids, atRA activates only RARs [11,33], whereas, 9cRA activates both RARs and RXRs [34].

Genetic studies have shown that RARα and RXRβ, are critical for spermatogenesis [35,36]. The testes from RARα knockout mice had a morphology similar to the VAD testis [35], whereas the RXRβ knockout mice were sterile because of abnormal spermiogenesis [36]. The RARβ, RARγ and RXRγ mutants were found to have normal spermatogenesis [37–39]. With respect to the RXRα mutant mice, they died in utero between embryonic days 13.5 and 16.4 and therefore did not live long enough to reveal whether RXRα is required for post-natal testis development and adult spermatogenesis [40].

It is clearly evident that retinoids are involved in the regulation of testicular functions, although the majority of research, to date have been conducted in rodents, while few information in animals showing a annual cyclic reproductive behaviour are available.

In this respect, the lizard, *Podarcis sicula*, shows a reproductive circannual cycle with spring full gonadal activity, complete summer regression and slow autumnal recrudescence without spermiation [41,42].

Specific changes in plasma sex-hormone levels occur during the different phases of the gonadal cycle [43,44]. Plasma androgens start increasing in February and peak in March, to reach their lowest values in June–July. 17β-Estradiol levels start increasing in May to become maximal in June–July. In autumn, estradiol levels are relatively high to decrease, thereafter, in late autumn–winter [42,44–48]. Moreover, in attempting to determine whether the use of retinoids affects testicular function also in other vertebrates, we have recently found that the use of all-*trans*-retinoic acid impairs spermatogenesis and enhances germ cell apoptosis in adult lizard [49].

Since the dynamic relationships between the elements involved in the control of lizard gonadal activity, as well as the mechanisms responsible for the action of the regulating factors, are not yet well defined, the goals of this study were to characterize the RARα, β and γ isoforms in the lizard and to investigate, by Northern blot and PCR analyses, the occurrence of retinoic acid receptor mRNAs in lizard testis, throughout the reproductive cycle.

2. Materials and methods

2.1. Animals

Sexually mature male lizards (*P. sicula*) were captured in the area surrounding Naples (Italy), during three phases of spermatogenetic cycle: full activity (May), summer regression (August) and autumnal recrudescence (October). Animals were maintained in terraria under natural photoperiodic and thermal regime to repair the effects of acute stress on gonadal hormone secretion [50], and fed on meal worms and fresh vegetables *ad libitum*. The lizards were sacrificed under anaesthesia and testes, liver and skin were removed, quickly frozen in liquid nitrogen, and stored at −80 °C for RNA extraction. Also, mice were used as positive control for RAR isoforms expression.

All the experiments were performed under approval of institutional committees (Ministero della Sanità); all efforts were made to avoid animal suffering and to miniminize the number of animals used.

2.2. Chemicals

All glassware were sterilized and RNAse free. All materials were molecular biology grade. α-^{32}P-dCTP (3000 Ci/mmol) was purchased from Amersham International (Amersham, Italy). Restriction enzymes were from New England Biolabs (Beverly, USA).