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Temporal Expression of Thyroid Hormone Receptor α1 in the Liver of the Lizard *Podarcis sicula*

FRANCESCA VIRGILIO[1], ROSARIA SCIARRILLO[*2], MARIA DE FALCO[1], RAFFAELLA COMITATO[1], VINCENZA LAFORGIA[1], LORENZO VARANO[1], AND ANNA CARDONE[1]

[1]*Dipartimento di Biologia Evolutiva e Comparata, Università di Napoli, Federico II, Napoli, Italy*

[2]*Dipartimento di Scienze Biologiche ed Ambientali, Università degli Studi del Sannio, Benevento, Italy*

ABSTRACT The effects of thyroid hormones on metabolism and development are mediated by thyroid hormone receptors (TRs). To gain a better understanding of the potential role of thyroid hormone receptors in the liver of the lizard *Podarcis sicula*, we have evaluated the expression of TRs during the more critical periods of the annual variations of thyroid activity. The results obtained have indicated that in the liver of the lizard *P. sicula* there are three transcripts: mRNA of 5.0 kb for TRα1, mRNA of 2.6 kb for TRα2, and 6.0 kb band, which represent unprocessed heteronuclear RNA, encoding unspliced primary transcripts of RNA prior to their processing into the mature TRα1 and TRα2. By means of slot-blot, we are able to determine that there is a change in the expression of TRs that occurs in the liver during the annual cycle of thyroid activity. A major expression registers in May, when the lizard thyroid gland shows the maximal activity. The combination of molecular biology with immunohistochemistry revealed that hepatic cells were also TRα IR positive. Particularly intense immunostaining was present in the cell nuclei of animals sacrified in May. These observations suggest that in lizard *P. sicula* the thyroid hormone (T3) might regulate hepatic activity, modulating TR mRNA levels. *J. Exp. Zool. 301A:212–217, 2004.*

INTRODUCTION

The thyroid gland and the hormones, 3, 3', 5–triiodo L-thyronine (T3) and L-thyroxine (T4), which it produces, are characteristic features of all vertebrates (Oppenheimer, '99). One striking characteristic of thyroid hormones is their ability to regulate a wide range of cellular functions in virtually every type of vertebrate tissue. They are known to exert profound effects on growth, development and differentiation, metabolism, and the maintenance of homeostasis (Oppenheimer et al., '91). However, much of the information on the functions of thyroid hormones and especially the molecular basis of their action, is largely restricted to mammals and amphibian metamorphosis (Shi et al., '94; Riberio et al., '98).

The biological effects exerted by thyroid hormones are mediated through specific nuclear receptors. Thyroid hormone receptors (TRs), as members of the large nuclear receptor superfamily, function as hormone dependent transcription factors (Riberio et al., '98). There are two isoforms of TRs, α and β, and each isoform has subtypes derived through alternative splicing or use of different promoters (Lazar and Chiu, '90).

Specific binding of thyroid hormones to nuclear receptors in mammals has been reported at a variety of cellular sites, such as the liver. The liver is an important target tissue for thyroid hormone action, even if the specific mechanisms by which thyroid hormones cross the blood sinusoidal membrane on hepatocytes, are not fully understood. Thyroid hormone receptors have been demonstrated in hepatic nuclei from several nonmammalian tetrapod vertebrates, including chicken embryos (Bellabarba and Lehoux, '81), the quail (Weirich and Mc Nabb, '84), and the bullfrog tadpole (Kistler et al., '75; Galton, '80). Studies on rainbow trout (Van Der Kraak and

*Correspondence to: Dr. Rosaria Sciarrillo, Dipartimento di Scienze Biologiche ed Ambientali, Università degli Studi del Sannio, Via Port'Arsa, 11– I–82100 Benevento, Italy. E-mail:sciarrillo@unisannio.it.

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Eales, ’80), coho salmon (Darling et al., ’82), lake trout (Weirich et al., ’87), and sea lamprey (Lintlop and Younson, ’83) have shown that the probable nuclear T3 receptors in the liver of these fish species have properties which very strongly resemble those of higher vertebrates.

Thyroid hormone receptors in lower vertebrates offer an opportunity to examine the evolution of a hormone receptor system, since the hormone molecules themselves have not changed during evolution.

In the seasonal lizard *Podarcis sicula*, the thyroid gland undergoes a marked annual cycle (Cavagnuolo et al., ’82), characterized by a functional stasis, starting in autumn to become full stasis in December-January. In this period, the follicular epithelium was low, while the colloid was compact and devoid of reabsorption vacuoles. In spring there was a thyroid activity resumption that reached its maximum in May-June; the follicular epithelium was very high and colloid was retracted with clear signs of reabsorption. The thyroid activity decreases again afterwards.

These annual variations in thyroid gland morphology are paralleled by variations in plasma levels of thyroid hormones. In fact, plasma concentrations of 3,5,3’–triiodo-L-thyronine (T3) and L-thyroxine (T4) increased rapidly at the beginning of spring, reaching peak levels in June; thereafter, they gradually decreased, reaching the lowest values in December (Sciarrillo et al., 2000).

The aim of the present study was to investigate the expression pattern of TRα isoform at selected months during the seasonal changes in thyroid activity. Therefore, we have evaluated the occurrence of TRα mRNAs in the liver by Northern-blot analysis and the expression of TRs in the RNA extracted from liver, by slot-blot technique. Besides the molecular analysis, we have carried out an immunocytochemical study to evaluate the distribution of TRα1–IR in the liver cells, during the critical period of thyroid activity.

MATERIALS AND METHODS

Animals and experimental design

We utilized adult specimens of lizards *Podarcis sicula*, a diurnal species living in fields. The animals were captured in the neighborhood of Naples (Italy) during critical periods for both thyroidal (Sciarrillo et al., 2000) and gonadal activities (Cardone et al., 2000, 2002): [a] March, to represent the beginning of activities, when the natural photoperiod is 11 h daylight with a temperature ranging from 12 to 14°C; [b] May, to represent the period of maximal activities and also the breeding time, when the natural photoperiod is 13 h daylight with a temperature ranging from 22 to 24°C; [c] October, to represent the inactive period for thyroid gland and gonads, when the natural photoperiod is 12 h daylight with a temperature ranging from 16 to 18°C.

Twenty animals were captured for each period. The animals were fed on fly larvae daily and fresh water was available ad libitum. They were maintained in terraria for two weeks under natural photoperiodic and thermal regime to repair the effects of acute stress. At the end of this period, the lizards were killed under anesthesia and the livers were aseptically excised. Some livers were frozen in liquid nitrogen for RNA extraction, the others were used for immunohistochemical analysis.

RNA extraction

Total cellular RNA was extracted following Chomczynski and Sacchi (’87), with minor modifications. The yield and quality of RNA were assessed by the 260/280 nm optical density ratio (1.93 ± 0.05) and by electrophoresis under non-denaturating conditions on 1.2% agarose gels.

Northern blot analysis of TRα expression

For Northern analysis, 30 μg of total RNA, isolated from livers of lizards captured in May, were subject to electrophoresis on 1.2% agarose-formaldehyde gel. Single strand (λ-DNA/Hind III digest, 23.130–0.564 kb, Stratagene, La Jolla, CA) and RNA molecular-weight markers (7.4–1.6 kb Boehringer) were used to determine the sizes. Samples and markers were transferred overnight onto nylon membrane (Nytran, Schleicher & Schuell) using 10X SSC (SSC: 0.015 M trisodium citrate, 0.15 M NaCl, pH 7.0). Filters were baked for 30 min/80°C in a vacuum oven and exposed to UV irradiation (254 nm/2 min). Prehybridization was performed at 42°C for 4h in 50% deionized formamide, 5X SSC, 0.1% SDS, 0.05 M phosphate buffer (ph 6.8), 0.005M EDTA, 5X Denhardt’s solution (Denhardt’s solution: 1% (w/v) ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA), 100 μg/ml yeast tRNA. Hybridizations buffer was as above. A 840 bp fragment (Pst1) from Xenopus TRα form cDNA (xTRa) was used as probe. Probe was labeled with a–^{32}P-dCTP by random priming to a specific activity of 5×10^8 cpm/μg. Hybridization was performed at 42°C overnight. Filters were washed twice with 2X SSC, 0.1% SDS at 68°C, twice with 0.2X SSC, 0.1% SDS at 68°C, and once with 0.1X SSC, 0.1% SDS at 72°C. Dried filters were exposed to X-ray film (Fuji HR-H) for 48–96h.

Slot-blot analysis of TRα expression

Total cellular RNA, isolated from lizard liver at critical periods of thyroidal activity (March, May, October) was subjected to analysis of the relative amounts of TR mRNA expression. For this purpose, the analysis of RNA was performed by quantitative slot-blot technique as described elsewhere (Varriale and Tata, '90; Varriale and Serino, '94). Thus, total RNA (15 μg/samples) was bound to nylon membrane (Nytran, Schleicher & Schuell) in a slot-blot apparatus (Schleicher & Schuell). Slot-blots were performed in duplicate and hybridized as described by Cardone et al. ('98). Prehybridizations, hybridizations, and washes were carried out as above. The relative amounts of TR mRNA were determined by densitometric scanning of the autoradiograms and normalized to corresponding values of β-actin mRNA.

TRα1 immunohistochemistry and quantitative analysis of positive cell numbers

TRα1 was detected using immunohistochemistry. The primary antiserum was raised in Santa Cruz Biotechnology. Inc. (Santa Cruz, CA) rabbit against a recombinant protein corresponding to aminoacids 1–408 of TRα1. In brief, livers were removed and fixed in Bouin's fixative for 24 h and subsequently washed in 75% ethanol overnight, dehydrated through graded ethanols, and embedded in Paraplast. Serial cross sections were cut at 5 μm and were processed for TRα1 immunohistochemistry, using the avidin-biotin-horseradish peroxidase technique (Vectastain, ABC kit; Vector laboratories, Burlington, CA). The sections were dewaxed and incubated with 3% H_2O_2 for 30 min to eliminate endogenous peroxidase activity. After blocking with 10% goat normal serum for 30 min, the sections were incubated overnight at 4°C with the primary antibody (diluition 1:500) in 0.01 M phosphate buffer saline containing 0.3% Triton X–100 and 3% normal goat serum. The sections were rinsed in PBS, incubated with biotinylated secondary antibody against rabbit IgG for 1h and subsequently with Vectastain ABC reagent for 1h. After being rinsed with PBS, the sections were incubated in 0.05 M TRIS HCl buffer (pH 7.2) containing 0.05% 3,3′–diaminobenzidine tetrahydrochoride and 0.01% H_2O. After 6–7 min, the reaction was stopped by several washes with 0.05 M TRIS HCl buffer (pH 7.2). The presence of TRα1 was detected with brightfield microscopy as dark reaction product in the cell nuclei. The control staining was performed using the preimmune serum from the same rabbit that produced the primary antibody. The numbers of TRα1 positive cells in the liver were counted in the different periods examined, using a digital imaging system (KS 300). Double-counting errors were corrected by the following formula proposed by Abercrombie ('46) to estimate nuclear populations from microtome sections. $P = A \times [M/(L+M)]$, with P being the corrected cell count, A the total cell count, M the section thickness (mm), and L the average diameter of the nucleus. Ten randomly selected TRα1 positive nuclei were measured with a microruler in each section and at least five sections were measured in each liver.

Statistics

All data are presented as means ± standard error of mean (SEM). Statistical analyses were performed by one-way analysis of variance (ANOVA) with repeated measures followed by Duncan's multiple range test for pairwise comparisons. Differences were considered significant if $P < 0.05$.

RESULTS

Northern blot analysis of TRα expression

In order to demonstrate the validation of the technique we have carried out a Northern blot detection of TRα mRNA from lizard livers of specimens captured during March (Fig.1). The probe used for TRα mRNA recognized both TRα1 and TRα2 forms. The molecular sizes of these transcripts were 5.0 kb for TRα1 and 2.6 kb for TRα2 mRNA. A minor 6.0 kb band was also observed; it presumably represents unprocessed heteronuclear RNA, encoding unspliced primary transcripts of RNA prior to their processing into the mature TRα1 and TRα2 transcripts. The filters were washed under stringent conditions, so it is evident that the liver of the lizard strongly expressed TRα mRNA.

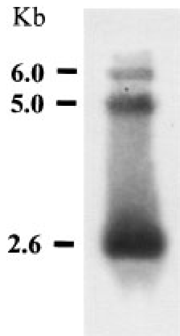

Fig. 1. Northern blot analysis of thyroid hormone receptor mRNA in lizard liver. Total RNA (30 μg), obtained from the whole liver, was subjected to electrophoresis under denaturating conditions and transferred to nylon membrane. The blot was hybridized with a random-labeled 840 bp fragment (Pst 1) from Xenopus TRα form (xTRα) or to 796 bp fragment (Hinf I) from Xenopus TRβÿform cDNA (xTRβ) (data not shown). The sizes of the specific TRα transcripts are indicated on the left side of the figure.

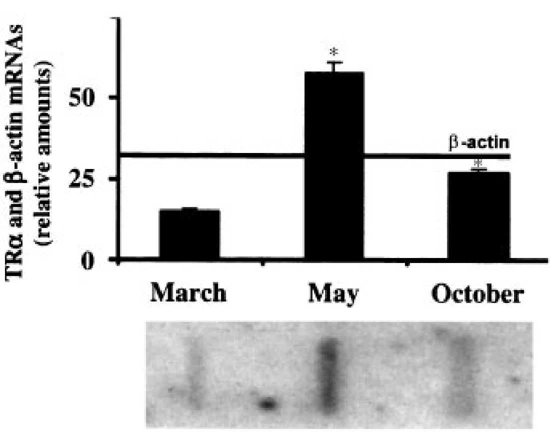

Fig. 2. Levels of TR mRNA expression in lizard liver between the different experimental periods. The autoradiogram represents a slot-blot hybridization of the TR mRNA. The relative concentrations were determined by densitometric analysis of the slot-blot hybridization and expressed as relative amounts of TR mRNA with respect to the total signals. Values are shown as means ± SD. *Significant at $P < 0.05$.

Levels of TR mRNA expression in lizard liver during the different periods

The levels of TRα mRNA expression in lizard liver during the different periods of thyroid activity are shown in Fig. 2. The densitometric values of TRα mRNA were obtained by means of scanning the hybridization signal for every sample and were expressed as quantity of signal. For this, the RNA samples of different periods of the year were loaded consecutively. The analysis of slot-blot hybridization, expressed as amounts of TRα mRNA, indicated that in the lizard liver, the TR mRNA levels were significantly increased during the periods of the year, showing low expression in March, highest expression in May and a reduction in October.

TABLE 1. Comparison of the distribution and relative density of TRα1 immunoreactivity in the liver of the lizard

Period	Relative density
March	+
May	+++
October	++

Density of TRα1 immunoreactivity : – none; + weak; ++ moderate; +++ strong.

TRα 1 immunohistochemistry

In order to confirm the data obtained by molecular techniques, the immunohistochemistry technique was used. Greater positivity of TRα 1 was evident in the liver cells in May than in October and March (Table 1). In particular, TRα1 immunoreactivity (IR) was distributed in the hepatocytes (Fig.3a); the reaction product was primarly confined to the cell nuclei.

No immunostaining was seen in the control sections incubated with pro-immune serum of the same rabbit that produced the primary antibody (Fig.3b).

DISCUSSION

Thyroid hormone regulates a wide range of biological processes across most animal species. Thyroid hormone receptors reside in the cell nucleus where they regulate transcription of specific genes (Utiger, '95; Riberio et al., '95, '98), and play a central role in the action of hormones. The present study indicates that the lizard *P. sicula* has a locus for TRα in the liver. The lizard liver expresses both TRα 1 and Trα 2 mRNA, having a molecular size of 5.0 and 2.6 Kb, respectively. This TRα expression is similar to that found in the lizard testis (Cardone et al., 2000).

We have demonstrated the variations in the expression of this receptor during some periods of the year. These modulations in their expression are in agreement with previous results that describe the annual profiles in plasma levels of thyroid hormones in *P. sicula* (Sciarrillo et al., 2000). In the lizard *P. sicula,* the thyroid gland releases both L-thyroxine (T4) and tri-iodo-L-thyronine (T3) into the bloodstream. In addition, a portion of the T3 present in the blood is

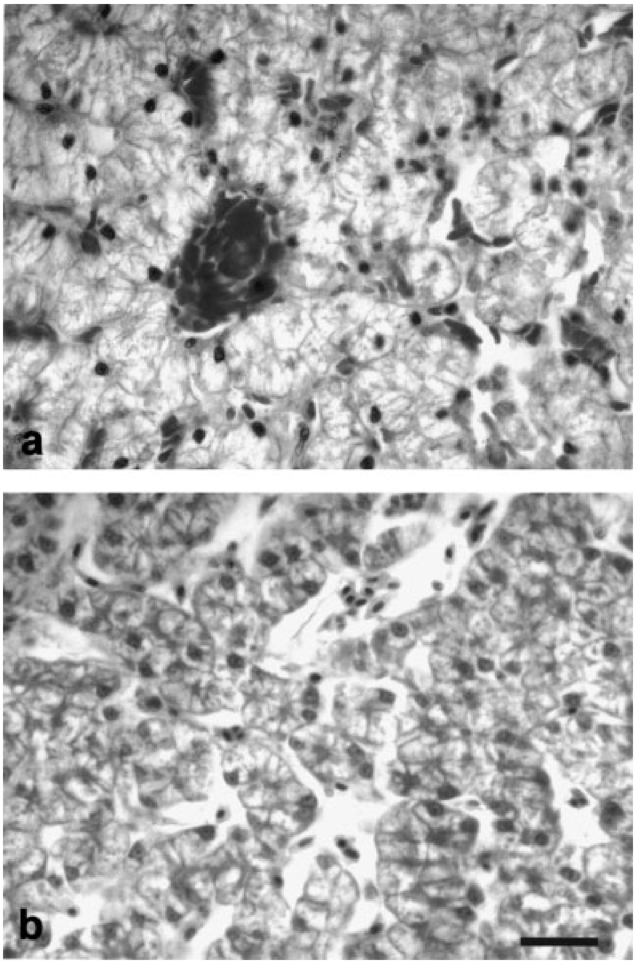

Fig. 3. Immunohistochemistry of TRα1 in lizard liver. **a**, The reaction products are primarly confined to the nuclei of the hepatocytes. **b**, Control section of the liver which underwent the same immunohistochemistry procedure except that the incubation was with pre-immune serum instead of the primary TRα1 antibody. Bar, 50 μm.

produced extrathyroidally, via the monodeiodination of T4 in peripheral tissues. Seasonal changes in thyroid function have been correlated with a number of reproductive events (spermatogenesis, ovulation, mating) in lizard *P. sicula*. Plasma concentrations of T4 and T3 exhibited profound variations during the year. They increased quickly at the beginning of spring, reaching peak levels in May. These levels gradually decreased (Sciarrillo et al., 2000). Therefore, this marked seasonality of plasma thyroid hormones in this lizard is correlated with a change of the expression of TR mRNA. The temperature, and of course the levels, of plasma T3 affected TR mRNA expression levels, indicating that T3 is able to up-regulate TR mRNA expression in lizard liver. The liver is an important target tissue for thyroid hormone action (Feng et al., 2000). It has been estimated that approximately 8% of the hepatic genes are regulated by thyroid hormones in vivo (Oppenheimer et al., '87); thus the liver is an ideal tissue in which to study gene regulation by thyroid hormones. This is the first time that periodic pattern of expression of TR in lizard liver has been demonstrated for a member of the nuclear receptor family. These results suggest an important correlation between seasonal changes of thyroid hormones and regulation of liver functions.

In order to confirm the pattern of TRα1 mRNA expression, we have investigated the distribution of TRα1 protein using immunohistochemistry. These studies demonstrated that TRα1–IR was located in the cell nuclei of hepatocytes. The number of TRα1–IR positive cells was much higher in specimens sacrificed in May than in March or October. Evidently, there is a pattern of expression of TRα1 in lizard liver: the TRα1 immunopositivity was low in March and increased in May; thereafter this immunopositivity gradually decreased in October.

These findings expand our knowledge on the liver distribution of TRα1. The wide distribution of this receptor in the liver is consistent with general knowledge about the thyroid hormone regulation on hepatic function.

T3, binding its receptor (TR) is able to influence the hepatic cells during the annual cycle of lizard activity, probably positively or negatively regulating transcription of target genes. Our studies indicate that the annual variations of TRα expression are correlated with the annual cycle of thyroid activity. This provides important information for understanding the mechanism through which thyroid cycles influence the hepatic activity.

In conclusion, our results clearly demonstrate that lizards express two different TRα isoforms (TRα1 and TRα2) and that there is an accumulation of TR mRNA in the liver in May, in correlation with seasonal peak of plasma thyroid hormone concentrations. Besides, the present data suggest that in lizards, seasonal increased T4 and T3 plasma concentrations might modulate hepatic activity, up-regulating TRα mRNA levels.

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