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Thyroid Hormone Receptors in Neonatal, Prepubertal, and Adult Rat Testis

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ABSTRACT: Thyroid hormone (TH) is involved in the differentiation and development of rat testis, whereas its role in adult testis function is still undefined. The aim of our work has been to further analyze the presence in the testis of rats of various ages of messenger RNA (mRNA) coding the different TH receptor (TR) subtypes using a sensitive assay, such as reverse transcriptase–polymerase chain reaction (RT-PCR). To rule out the possibility of an ''illegitimate transcription,'' we have analyzed both T_3-binding capacity of adult rat testis and the presence in the same organ of TR proteins by immunohistochemistry, using specific antibodies directed against the various TR isoforms. Messenger RNA coding for TR α1 and α2 isoforms was clearly visible in gels prepared from RT-PCR samples obtained from the testis of rats of all ages, including adults, whereas mRNA for the TRβ1-β2 was absent. The T_3 maximal binding capacity (C_{max}) by nuclear extracts of testicular homogenates gradually decreased from birth to adulthood, still remaining significantly detectable in adult testis, and represented approximately 1% of the C_{max} observed in the liver. The immunostaining technique revealed an intense nuclear staining along the basement membrane of testicular tubules prepared from rats of all ages and incubated with an antipeptide antibody specific for TRα1 (α1-403). Staining with an antipeptide antibody specific for TRβ1 (β-62) was never present. Our data show that mRNAs coding for the functional TRα1, and also for the still undefined α2, are present in the testis of rats of all ages. T_3-binding activity and immunohistochemical studies confirmed that the message is translated into proteins. The transcriptional activity clearly decreased from birth to adulthood, but it still remained significantly present. The presence of a TRα1 message indicates that the adult rat testis may be directly responsive to T_3 and, therefore, suggests an action of TH on rat testis that is not only developmental, but also metabolic.

Key words: Sertoli cells, thyroid hormone, thyroid receptors, mRNA.

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The relationship between thyroid and testicular function in the rat is still controversial. The testis was considered for years to be a thyroid hormone (TH)-unresponsive tissue (Barker and Klitgaard, 1952). However, various recent studies have shown that TH plays an important role in rat testis development. In fact, short-term experimental hyperthyroidism induces Sertoli cell differentiation (Jannini et al, 1993), whereas neonatal transient hypothyroidism causes a delayed differentiation of Sertoli cells, an increase in their number, and thus, an increase in the number of germ cells because the ratio of Sertoli cells/germ cells is constant (Van Haaster et al, 1992; Hess et al, 1993). The ultimate consequence of this condition is an enlargement of adult rat testis. A similar condition has also been observed in human juvenile hypothyroidism, supposedly for the same reasons (Jannini et al, 1995). Recent data also suggest a regulatory role of TH in the differentiation of Leydig cells in the neonatal rat testis (Mendis-Handagama et al, 1998).

The biological effects induced by TH are mediated by specific nuclear receptors (TRs). Various subtypes of TRs have been demonstrated: α1, α2, β1, and β2. The functional TRs are α1, β1, and β2, the latter being mainly confined to pituitary and hypothalamus. The ontogenetic pattern of the expression of the various TR subtypes during rat testis development was studied by Jannini et al (1994) using an RNAse protection assay. Alpha1 messenger RNA (mRNA) expression was maximal during the perinatal period and subsequently declined until day 60, when it was no longer detectable, showing a pattern of expression parallel to that of the T_3-binding capacity of nuclear extracts (Jannini et al, 1990). The β1 message was never detected, whereas the α2 message was always present from the perinatal period throughout adult life. Similar results were obtained by Strait et al (1990). On the other hand, another report has claimed the presence of TRβ1 mRNA in Sertoli cells isolated from prepubertal rat and piglet testis using a reverse transcriptase–polymerase chain reaction (RT-PCR) assay (Palmero et al, 1995). A

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thorough analysis of published data, however, shows that T_3-binding capacity is not completely absent in adult rat testis, but in fact is present at the significant level of approximately 1% of that found in the liver (Oppenheimer et al, 1974). Even Jannini et al (1994) have shown a weak band corresponding to the TRα1 mRNA in Sertoli cell–enriched culture from adult rats using an RNAse protection assay.

The aim of our work has been to further analyze the presence in the testis of rats of various ages of mRNA coding the different TR subtypes using a more sensitive assay, such as RT-PCR. To rule out the possibility of an ''illegitimate transcription,'' we have analyzed both T_3-binding capacity of adult rat testis and the presence in the same organ of TR proteins by immunohistochemistry, using specific antibodies directed against the various TR isoforms.

Materials and Methods

Animals

Male Wistar rats were used in all experiments. The animals were killed under ether anesthesia. Decapsulated testes were obtained from 5-, 20-, 40-, and 60-day-old rats. The number of testes used at each age was optimized to obtain sufficient tissue for RNA isolation and T_3-binding analysis.

RNA Isolation

Total RNA (tRNA) was prepared by using RNAzol (Cinna/Biotecx Laboratories, Houston, Tex) according to the manufacturer's instructions. The concentration of the tRNAs and the efficacy of the extractions were determined by optical density readings at 260 and 280 nm and verified by ethidium bromide staining. Only RNAs showing 2 clear bands of 28S and 18S corresponding to ribosomal RNAs were selected for further studies.

Reverse Transcription and Complementary DNA Amplification

Five micrograms of tRNA were mixed with 5 μg of random oligonucleotide primers (Pharmacia, Piscataway, NJ) for 10 minutes at 65°C, and incubated with 1000 IU Moloney murine leukemia virus (M-MLV) RT (Promega, Madison, Wis) in the presence of 10 μL deoxy-NTP (10 mmol/L each) for 1 hour at 42°C. Five microliters of the complementary DNA (cDNA) obtained by RT were amplified by PCR in a thermal cycler (Mj Research PTC-200; Pelfier Thermal Cycler, Genenco, Florence, Italy), employing 4 pairs of primers, 1 common and the other 3 specific for α1, α2 (and α3), and β1 (and β2) TR isoforms (Life Technologies, S Giuliano Milanese, Italy; Table 1).

PCR reaction was carried out in a final volume of 50 μL containing 25 pmol of each primer, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, and 2.5 IU of *Taq* DNA polymerase (Promega). The amplification profile involved denaturation at 94°C for 1 minute, primer annealing at 62°C for 1 minute, and extension at 72°C for 90 seconds. Amplification was allowed to proceed for 30 cycles. The size of the PCR products was analyzed by 2% agarose gel electrophoresis stained with ethidium bromide. One set of human β-actin primers was used as a positive control.

Table 1. *Sequences of specific and common primers used in the RT-PCR experiments**

TR	Sequence (5′ → 3′)	Position
α1	CAG GCT CCC CCT CAC CAG CT	1776–1795
α2	GTG GAG CAG CTC CAG GAG ACG	1381–1402
β1	GGC TAA CAG GAA ACT GTC TTG G	1244–1265
Common	AGG CTG CTG CAT GGA GAT CAT GTC	756–779†
		903–926‡

* RT-PCR indicates reverse transcriptase–polymerase chain reaction; TR, thyroid hormone receptor.
† Position in TR α isoform.
‡ Position in TR β isoform.

Analysis of T_3-Binding Capacity

The analysis of the binding of T_3 to nuclear extracts from rat testes and liver of various ages was performed using the method of Torresani and DeGroot (1975). Briefly, testes and liver were rapidly removed, weighed, and gently homogenized in 3–4 vol of 0.32 mM sucrose + 2 mM $MgCl_2$ (0.32 SM) with a Teflon pestle in a glass homogenizer. The solution was washed twice by centrifugation at 1000 × *g* for 10 minutes. The pellet was resuspended with a solution of 2 M sucrose + 2 mM $MgCl_2$ and centrifuged at 100 000 × *g* for 45 minutes and then submitted to extraction conditions.

Nuclear extracts were incubated in 0.3 mL of a buffer solution (20 mM Tris-HCl, 2 mM $MgCl_2$, 0.1 M NaCl, and 2 mM dithiothreitol) with 0.01 pmol ^{125}I-T_3 and increasing concentrations of cold T_3 for 3 hours at room temperature.

Protein-bound T_3 was isolated from unbound T_3 by adding 0.3 mL of a Dowex-1 (Cl^-, 1 × 8, 200–400 mesh) anion exchange resin suspension to each test tube. After shaking for 10 minutes at 0°C and then centrifuging, radioactive measurements were performed on aliquots of the supernatant. Specific T_3 binding refers to that inhibited by a 1000-fold molar excess of nonradioactive T_3 in the incubation medium. All experiments were performed in duplicate. Scatchard plot analysis was performed to determine binding parameters (K_a and C_{max}) by using the Ligand program (developed by Munson and Rodbard). Results were expressed as both femtograms of T_3 bound per 10^6 nuclei and as ng/mg DNA, in order to compare them with the data previously reported by Jannini et al (1990) and Oppenheimer et al (1974), respectively.

Immunohistochemistry

Rat testes were immediately removed, embedded in OTC, frozen in liquid nitrogen, and stored at −80°C. Six-micron-thick sections were cut with a cryostat and placed on glass slides. The slides were fixed in Zambonis fixative (picric acid paraformaldehyde solution buffered with phosphate) for 5 minutes at room temperature. The slides were then washed in phosphate-buffered saline (PBS) pH 7.3, incubated for 45 minutes with a blocking solution (PBS + 1% bovine serum albumin + 10% goat serum), and incubated for 2 hours with 2 antipeptide antibodies directed

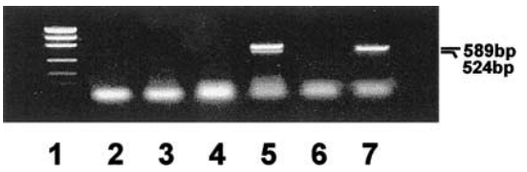

Figure 1. Specific PCR amplification of the various TR isoform cDNAs in rat adult testis. The cDNA transcribed from total mRNA was used for the PCR amplification of TRs. Primers α1 and the common primer amplify rTRα1 (lane 7). Primers α2 and the common one amplify c-erb-A α2 and α3 (lane 5). Primers β1 and the common one fail to amplify the corresponding receptor (lane 3). Lanes 2, 4, and 6 represent the negative controls. The products correspond to the expected size.

against TRs and affinity-purified with c-Erb-A α or β, diluted 1:100 and 1:300 in 0.1% BSA-PBS. The antigen-antibody complexes were visualized using a sensitive avidin-biotin-immunoperoxidase method (Vectastain ABC; Vector, Burlingame, Calif) and 3,3′-diaminobenzidine as substrate (Macchia et al, 1992).

The antibodies used were α1-403 directed against the carboxy-terminal portion specific for TRα1 and β-62, which reacts against an epitope in the A/B domain specific for the TRβ1. Immunoglobulins from preimmune sera of the same rabbits used to raise antibodies were employed as negative controls. The preincubation of the antibodies with their respective peptides was carried out as a further control. The characterization of these antipeptide antibodies, competition studies, and immunoprecipitation analysis were reported previously (Macchia et al, 1990, 1992; Falcone et al, 1994).

Results

TR mRNAs

To evaluate the expression pattern of TRs in the rat testis we performed an RT-PCR analysis on neonatal, prepubertal, and adult tissue. Messenger RNA coding for TRα1 isoform was clearly visible in gels prepared from RT-PCR samples obtained from the testis of rats of all ages, including adults. The same band was obtained in liver, used as a control tissue. The α2 message was also expressed in all tissues examined from rats of all ages. In contrast, the β message was always absent in the testis, but present in liver (data not shown). Figure 1 shows the results obtained in adult rat testis using specific primers for rat TRα1, α2, α3, and TRβ1, and a primer common for all the isoforms.

T_3-Binding Activity

Nuclear extracts from rat testis homogenates were incubated with radioactive and cold T_3 in order to evaluate the T_3 binding capacity. Our experiments show that the T_3 C_{max} by nuclear extracts of testicular homogenates decreases consistently from birth to adulthood, still remains significantly detectable in adult testis, and represents approximately 1% of that found in the liver. The affinity constant (Ka) was almost unchanged in rats of all ages (Table 2).

Table 2. *T_3 binding studies on testicular homogenates from rats of various ages: evaluation of Cmax and Ka**

Tissue	Cmax	Ka (10^{-9} M)
20-day-old-rat testis	0.0068† 40.8‡	3.22
40-day-old-rat testis	0.0021† 12.6‡	5.23
60-day-old-rat testis	0.0012† 8.5‡	3.51
Rat liver	0.113† 680‡	5.95

* T_3 indicates triiodothyronine; Cmax, maximal binding capacity; ka, affinity constant.

† Cmax in nanograms of T_3 per milligram of DNA according to Oppenheimer et al (1974).

‡ Cmax in femtograms of T_3 per 10^6 nuclei according to Jannini et al (1994).

Immunohistochemistry

The immunostaining technique revealed an evident nuclear staining in testicular tissue prepared from rats of all ages and incubated with the α1-403 antibody. Staining with β-62 antibody was absent. Staining was also absent with preimmune sera and when the first antibody was omitted, whereas it was barely visible when the antibodies were preincubated with their respective peptides. Although the cellular types stained were not clearly distinguishable because of the freezing procedure, nuclear staining was nevertheless present in the basal compartment of the tubules, indicating the presence of TR in Sertoli and germinal cells (Figure 2).

Discussion

It is well known that TH plays an important role in the development and differentiation of most tissues. This role has also been demonstrated in rat testis. In fact, transient hypothyroidism in neonatal rats induces a proliferation of Sertoli cells and delays their differentiation (Francavilla et al, 1991; Van Haaster et al, 1992; Hess et al, 1993). It seems that a critical time window exists for TH to exert its effects on testis development. TH action is supposed to be mediated by specific TRs present in Sertoli cells, whereas the nontubular fractions of rat testis are devoid of any T_3-binding capacity (Jannini et al, 1990; Cooke et al, 1991). These findings also seem to be confirmed in humans, in whom juvenile transient hypothyroidism causes a testicular enlargement without an increase of androgen production, due to the nonactivation of the Leydig cells (Laron et al, 1970; reviewed by Jannini et al, 1995).

Our data show that mRNAs coding for the functional

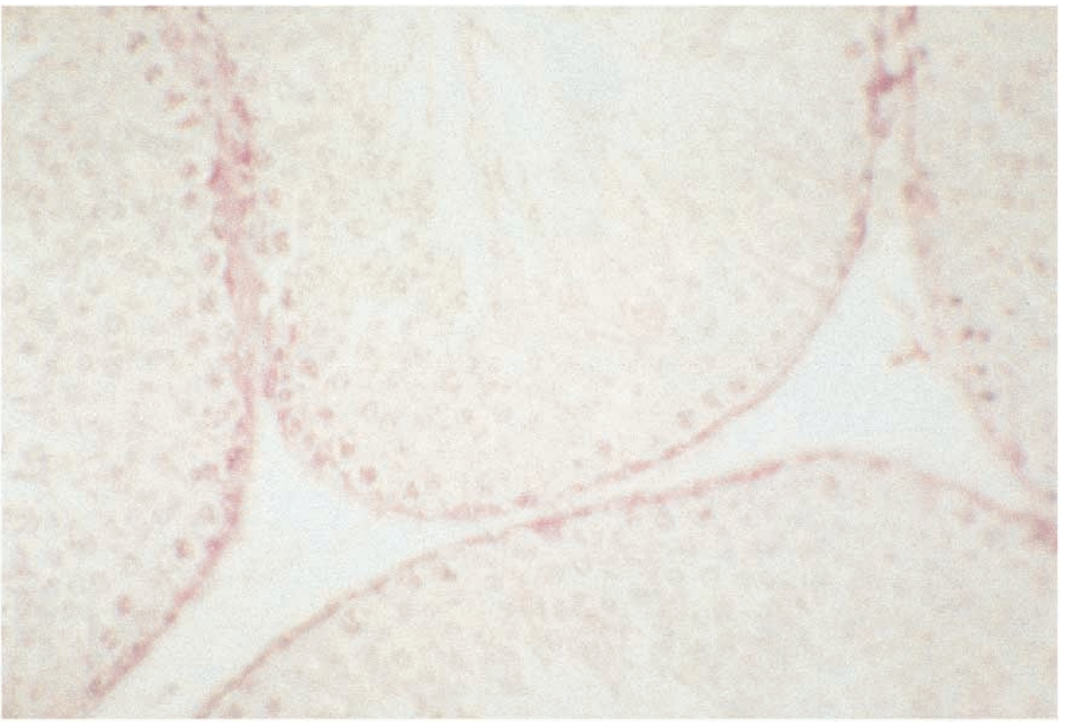

Figure 2. Nuclear TRα1 immunoreactivity in 60-day-old rat. The staining intensity is strong in the basal layer of tubular cells both in Sertoli cells and spermatogonia.

TRα1, and also for the orphan TRα2, are present in the testis of rats of all ages. T_3-binding activity and immunohistochemical studies confirmed that the message is translated into protein. The transcriptional activity clearly decreases from birth to adulthood, but it still remains significantly present. The presence of TRα1 mRNA and protein indicates that the adult rat testis may be directly responsive to T_3 and, therefore, suggests an action of TH on rat testis, which affects not only differentiation and development, but also the metabolism of tubular cells.

Indeed, the presence of TRs, and therefore the direct biological effects of TH in the adult female gonad, both in humans and experimental animals, is well established (Zhang et al, 1997). TH synergizes in vitro with both human chorionic gonadotropin (Goldman et al, 1993) and follicle-stimulating hormone (Maruo et al, 1987) in stimulating granulosa cells and estrogen production.

Similarly, we can hypothesize a modulating function of TH on the relationship between Sertoli cells and germ cells. In fact, although detailed studies on adult Sertoli cells have not been performed, TH has been shown to stimulate prepubertal Sertoli cells to uptake glucose (Ulisse et al, 1992) and to secrete substances such as lactate, which is essential for germ cell survival; and growth factors, such as IGF-1, which stimulate DNA synthesis in mitotic germ cells (Palmero et al, 1990). The results of our immunohistochemical studies indicate the presence of TRα1 not only in Sertoli cells but also in spermatogonia. Previous studies (Jannini et al, 1995) demonstrated an increased production of sperm in hypothyroid rats treated with TH. In view of our data this phenomenon could now be explained as being a direct effect of TH on the differentiation of spermatogonia to initiate the maturative cycle.

The very low T_3-binding capacity of nuclear extracts from adult rat testis homogenates could be explained by a progressive down-regulation of the expression of TRs, or by the fact that the tubular compartment constitutes about 80%–90% of the entire weight of rat testis, but Sertoli cells and spermatogonia represent just 10%–20% of the total number of tubular cells (Wing and Christensen, 1982).

Even though TH has been shown to up-regulate the postnatal Sertoli cell androgen receptor mRNA (Arambepola et al, 1998), the absence of a direct action on Leydig cells could explain why acute changes of circulating TH fail to cause definite modifications of gonadal function in the adult. The bulk of our data seems to indicate a direct action of TH only on spermatogenesis, which represents a slow and long process aimed at giving rise to millions of mature spermatozoa (Wing and Christensen, 1982). Indeed, some reports have been published concerning the effects of long-term hyperthyroidism and hypothyroidism on spermatogenesis and fertility, but no

well-defined controlled studies have yet addressed this question (Jannini et al, 1995).

Recent studies on transgenic mice lacking either TRβ1 or TRα1 (Forrest et al, 1996; Weiss et al, 1997; Wikstrom et al, 1998) have clearly shown a different effect of TH on the same tissues, depending on which type of TR is present. In the light of our findings, both developing and adult rat testis seem to represent a physiological model of an organ devoid of the TRβ isoform in which TH may act only on the genes responsive to the TH-TRα1 complex.

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