Type 2 iodothyronine deiodinase is highly expressed in germ cells of adult rat testis

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Abstract

The testis has been classically described as a thyroid hormone unresponsive tissue, but recent studies indicate that these hormones might play an important role in developing testes. We have previously demonstrated that type 2 iodothyronine deiodinase (D2), a thyroid hormone-activating enzyme, is expressed in adult rodent testis and that its activity is induced by hypothyroidism. Nevertheless, the precise location of D2 in testis is not known. The aim of the present work was to determine the testicular cell types in which D2 is expressed using real-time PCR analysis, *in situ* hybridization histochemistry, and determination of D2 activity in cell fractions isolated from adult euthyroid and/or hypothyroid rat testis. The D2 mRNA levels in germ cells were higher than those from somatic cells (6.94 ± 1.49 vs 2.32 ± 0.79 arbitrary units (au); $P=0.017$). Hypothyroidism increased D2 expression in germ cells (6.94 ± 1.49 vs 8.78 ± 5.43 au, $P=0.002$) but did

not change D2 transcripts in somatic cells significantly (2.12 ± 0.79 vs 2.88 ± 1.39 au, $P=0.50$). *In situ* hybridization analysis showed that D2 mRNA is specifically present in elongated spermatids undergoing differentiation, whereas other germ cell types and Sertoli cells of seminiferous epithelium and the interstitial cells were virtually negative for this enzyme. The enzyme activity measured in germ and somatic isolated cell fractions (0.23 ± 0.003 vs 0.02 ± 0.013 fmol/min per mg protein respectively; $P<0.001$) further confirmed the real-time PCR and *in situ* hybridization results. Hence, our findings demonstrated that D2 is predominantly expressed in elongated spermatids, suggesting that thyroid hormone might have a direct effect on spermatogenesis in the adult rats.

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Introduction

The iodothyronine deiodinases types 1, 2, and 3 (D1, D2, and D3) constitute a family of oxidoreductases that catalyze the removal of iodine from the outer ring (D1 and D2, activation) or inner ring (D3, inactivation) of the thyroid hormones (Bianco *et al.* 2002). All the three deiodinases contain the uncommon amino acid selenocysteine in their active site, an essential residue for efficient catalysis (Berry *et al.* 1992, Croteau *et al.* 1995, Salvatore *et al.* 1995). Thyroxine (T₄) activation to tri-iodothyronine (T₃) via D2 allows for changes in intracellular thyroid status in a tissue-specific manner independent of serum T₃. This system has been extensively characterized in the brain, pituitary, and brown adipose tissue

(BAT). Recent studies suggest that D2 also contributes to a significant fraction of plasma T₃ in rodents and humans (Maia *et al.* 2005, Streckfuss *et al.* 2005, Schneider *et al.* 2006). Although several factors such as cold, fasting, and hormones influence deiodinases activities, their main regulator is thyroid status. Hypothyroidism results in an increase of D2 activity and opposite changes are noted in hyperthyroidism (Bianco *et al.* 2002).

The testis was considered for years to be a thyroid hormone unresponsive organ, a concept based on data showing minimal levels of testicular thyroid hormone receptors (TRs) and lack of thyroid hormone effects on oxygen consumption in adult rat testes (Oppenheimer *et al.* 1974). However, clinical reports and experimental studies performed

during the last decades have indicated that thyroid hormones might have critical effects of this organ, especially during development (Jannini *et al.* 1995, Sakai *et al.* 2004). Abnormalities on reproductive hormone production, ejaculated volume, sperm motility, and structural morphology of rat and human testes have been associated with altered thyroid hormone status (Buzzard *et al.* 2000, Carani *et al.* 2005).

Indeed, experimental evidence supports the concept that thyroid hormones are important for testicular development and function. The ontogenic pattern of expression of the various TR subtypes in human and rat testes has been studied. TR α 1 isoform was shown to be present in testis throughout development, from fetal life to adulthood, being maximally expressed in the perinatal period in both species (Buzzard *et al.* 2000, Jannini *et al.* 2000, Canale *et al.* 2001). Recently, a detailed testis morphological analysis was performed on *rdw* mutant rats that have congenital hypothyroidism due to a missense mutation in the thyroglobulin gene (Sakai *et al.* 2004). The *rdw* testis requires a longer time to develop and shortly after full maturation, the normal structure begins to degenerate, indicating that thyroid hormone plays a role in developing and maintaining normal testicular function. Moreover, determination of the expression profile of the deiodinases demonstrated that D2, a T₄-activating enzyme, is present in rat testis from fetal to adult life (Bates *et al.* 1999) and that testis D2 activity is highly sensitive to hypothyroidism (Wagner *et al.* 2003). Nevertheless, the exact cellular localization of D2 expression in this organ remains unclear.

The present study was undertaken to further analyze the D2 expression in adult rat testis. We aimed to determine the testicular cell subtypes in which this enzyme is expressed using a combination of real-time PCR analysis, *in situ* hybridization, and D2 activity in somatic and germ cell fractions. D2 expression in other tissues of male reproductive system of rats was also evaluated.

Material and Methods

Animals

The experiments were performed on 100- to 120-day-old male Wistar rats, housed under conditions of controlled lighting and temperature, fed a commercial diet and water was available *ad libitum*. For real-time PCR and deiodinase assays, rats were made hypothyroid by administering 0.03% methimazole (MMI; Biolab, SP, Brazil) in the drinking water for 4 weeks to reduce their plasma T₄ levels. Control rats received regular drinking water *ad libitum*. Euthanasia was performed in a CO₂ chamber and tissues were rapidly removed, frozen in liquid nitrogen, and stored at -70 °C for subsequent D2 activity determination. For isolation of cell fractions, testes were removed, decapsulated, and immediately used. All animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian

Veterinary Medicine Council and the Brazilian College of Animal Experimentation.

Real-time PCR analysis

RNA was reverse transcribed with the SuperScript Preamplification System for First Strand cDNA Synthesis (Invitrogen Corp.) using 3 µg total RNA and 100 ng random hexamers. Reactions for the quantification of target mRNAs were performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Warrington, UK) using the SYBR Green PCR Master Mix (Applied Biosystems) and cyclophilin as a housekeeping internal control. Samples were run in duplicate. The cycle conditions were 94 °C × 5 min (Hot Start), 40 cycles of 94 °C × 30 s; 58 °C × 30 s; 72 °C × 45 s and a final 1 min extension period. Initially, standard curves representing five-point serial dilution of mixed cDNAs of the control and experimental groups were analyzed and used as calibrators to determine the relative quantification of product generated in the exponential phase of the amplification curve. Comparable efficiency was observed presenting $r^2 > 0.99$. Sample quantification was calculated by the standard curve and corrected by the internal control cyclophilin in all experiments. Oligonucleotides for rat D2 and cyclophilin were (5'-TTCTCCAAGTGCCTC-TTCCTG-3' and 5'-CCCATCAGCGGTCTTCTCC-3') and (5'-GCCGATGACGAGCCCTTG-3' and 5'-TGCCGCCAGTGCCATTATG-3') respectively.

In situ hybridization histochemistry

Male euthyroid adult rats were anesthetized with sodium pentobarbital (50 mg/kg body weight i.p.), blood was taken from the inferior vena cava and the animals were immediately perfused transcardially with 20 ml 0.01 M PBS (pH 7.4) containing 15 000 IU/l heparin sulfate followed by 150 ml of 4% paraformaldehyde in PBS. The testes were removed, post-fixed by immersion in the same fixative for 2 h at room temperature and then cryoprotected in 20% sucrose in PBS at 4 °C overnight. The testes were then placed in a cryomold, covered with optimal cutting temperature (OCT) compound (Tissue Tek, Nussloch, Germany) and snap frozen on dry ice. Serial 14 µm thick coronal sections were cut on a cryostat (Leica CM3050 S, Leica Microsystems GmbH, Nussloch, Germany) and adhered to Superfrost/Plus glass slides (Fisher Scientific, Pittsburg, PA, USA). The tissue sections were desiccated overnight at 42 °C and stored at -80 °C until prepared for *in situ* hybridization histochemistry.

Methods for double-labeling *in situ* hybridization have been described in detail elsewhere (Kakucska *et al.* 1992, Tu *et al.* 1997). Tissue sections were hybridized with an 800 bp single-strand [³⁵S]-UTP-labeled cRNA probe complementary to the entire coding region of the rat *Dio2* gene. Briefly, the hybridization was performed under plastic coverslips in a buffer containing 50% formamide, 2 × standard

sodium citrate ($2\times\text{SSC}$), 10% dextran sulfate, 0.5% sodium dodecyl sulfate, 250 $\mu\text{g/ml}$ denatured salmon sperm DNA, and 6×10^5 c.p.m. of radiolabeled probe for 16 h at 56°C . Slides were dipped into Kodak NTB2 autoradiography emulsion (Eastman Kodak) and the radiograms developed after 6 days of exposure at 4°C . After being cleared in graded solutions of ethanol and HistoSol (National Diagnostics, Atlanta, GA, USA), the sections were stained or not with haematoxylin and eosin, coverslipped in HistoMount (National Diagnostics), and the radiograms visualized and photographed under bright- or dark-field illumination with a Zeiss III (Carl Zeiss Inc., Thornwood, NY, USA) or Provis AX-70 Olympus (Olympus America Inc., Lake Success, NY, USA) microscope.

High-resolution light microscopy (HRLM)

Since frozen sections subjected to *in situ* hybridization analyses are very thick ($\sim 14\ \mu\text{m}$) and show low resolution, we used a high-resolution approach to identify fine details of germ and Sertoli cells under light microscopy (Chiarini-Garcia & Russell 2001). Staging of the spermatogenic cycle was previously determined, based on the youngest generation of spermatids and development of their acrosomal system. Fifteen minutes after i.p. heparin injection (125 IU/kg body weight), rat testes were briefly washed with saline by perfusion via the cardiac route and subsequently perfused-fixed with glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4). After that, testes were removed and sliced in small slabs that were rinsed overnight in 0.05 M cacodylate buffer and post-fixed in 1% osmium: 1.25% potassium ferrocyanide mixture, dehydrated in a graded series of ethanol, infiltrated, and embedded in Araldite. From the araldite-embedded blocks, $1\ \mu\text{m}$ thick sections were obtained with a Reichert Jung microtome (model 1140/autocut, Tissue Tek) and stained with toluidine blue-borate for further analysis at a BX-41 Olympus microscope (Olympus). Photomicrographs were taken with a Q-color 3 digital camera (Olympus).

Isolation of cell fractions

Testicular cell fractions of euthyroid and hypothyroid rats were prepared as previously described (Gelain *et al.* 2003). Briefly, testes were removed, decapsulated, and enzymatically digested with 30 ml trypsin (Sigma–Aldrich) and 250 μl DNase (Sigma–Aldrich) for 15 min at 37°C . This homogenate was centrifuged for 5 min at 400 g and the pellet was washed with 1% soybean trypsin inhibitor (Sigma–Aldrich) in complete Hanks balanced salt solution (HBSS). Cells were then centrifuged for 5 min at 400 g and the pellet was collected and incubated with 20 ml collagenase (Sigma–Aldrich) for 20 min at 37°C . This homogenate was centrifuged for 5 min at 400 g and the pellet was resuspended with HBSS and filtered in a 100 μm diameter nylon mesh

(Sigma–Aldrich) in order to separate germ from somatic cells. Cell fractions were immediately used for the enzymatic assay as described below.

Deiodinase assay

D2 assay was performed as previously described (Wagner *et al.* 2003) for both euthyroid and hypothyroid groups. Tissue samples were homogenized and sonicated on ice in buffer containing $1\times\text{PE}$ (0.1 M potassium phosphate and 1 mM EDTA), 0.25 M sucrose, and 10 mM dithiothreitol (DTT; Invitrogen), pH 6.9. The reaction mixtures containing 100–250 μg tissue proteins were incubated in a total volume of 300 μl with 100 000 c.p.m. of $\text{L}-[^{125}\text{I}]\text{T}_4$ (Amersham) purified by LH-20 sephadex column (Pharmacia); 1 nM unlabeled T_4 , 20 mM DTT, and 1 mM propylthiouracil (PTU; Sigma–Aldrich) in PE buffer at 37°C for 2–3 h. Reactions were determined by the addition of 200 μl horse serum and 100 μl 50% trichloroacetic acid. After centrifugation at 12 000 g for 3 min, free ^{125}I in the supernatant was counted with a gamma counter. The K_m/V_{max} determinations were performed with 0.25, 0.5, 1, 3, and 10 nM unlabeled T_4 . Activity is expressed as femtomoles iodide generated/min per mg protein. All reactions were performed in duplicates or triplicates and the experiments were repeated twice.

Statistical analysis

Data were analyzed using PRISM software (GraphPad Software Inc., San Diego, CA, USA) and results were shown as means $\pm$ s.d. Four to six animals were used per group per experiment. Comparisons between two groups were performed using student's *t*-test. $P<0.05$ was considered statistically significant.

Results

Real-time PCR analysis

D2 transcript levels were evaluated in germ and somatic cells isolated from euthyroid and hypothyroid rat testis by real-time PCR. Although D2 mRNA was detected in both germ and somatic cells, D2 mRNA levels were significantly higher in germ than in somatic cells (6.94 ± 1.49 vs 2.32 ± 0.79 arbitrary units (au) respectively; $P=0.017$). Hypothyroidism induced a significantly increase in D2 expression in germ cells (6.94 ± 1.49 vs 8.78 ± 5.43 au, $P=0.002$) but did not change D2 mRNA levels in somatic cells significantly (2.12 ± 0.79 vs 2.88 ± 1.39 au, $P=0.5$).

Cellular localization of D2 transcripts in rat testis

To address the specific localization of D2 within germ cells of the seminiferous epithelium, we performed *in situ*

Figure 1 *In situ* hybridization histochemistry autoradiograms of type 2 iodothyronine deiodinase (D2) in rat seminiferous epithelium. Dark- (A) and bright- (B) field microscopy shows identical fields of part of the seminiferous epithelium (longitudinal sections) with intense labeling for D2 mRNA in spermatids at stages III/IV and VII/VIII of the cycle. In (C), cross-sectioned seminiferous tubules at different stages of the cycle (III/IV, V, VII, and VIII) show D2 labeling coinciding with the localization of spermatids. Note that these cells are in differentiation process and occupy different heights of the seminiferous epithelium. Bars represent ~50 μ m.

hybridization histochemistry in testes from euthyroid adult rats. Evaluation of the seminiferous epithelium at dark- and bright-field microscopy showed that spermatids were intensely labeled for D2 mRNA (Fig. 1A). This cell type could be observed at different heights in the seminiferous epithelium because of the differentiation process when

round spermatids become spermatozoa (spermiogenic phase; Chiarini-Garcia & Russell 2001). Remarkably, we observed that elongated spermatids were consistently and preferentially labeled for D2. For instance, at stage III/IV of the spermatogenic cycle, when elongated spermatids are localized more internally in the seminiferous epithelium,

labeling for D2 mRNA was observed at the same cell position (Fig. 1B and C). At several other stages of the cycle, a dense D2 labeling coincided with the localization of more mature elongated spermatids (Fig. 1C). Control hybridization (D2 sense probe) was negative (data not shown).

For precise identification of cycle staging, we next compared *in situ* hybridization sections with high-resolution sections from rat testes. D2 labeling corresponded to the position of elongated spermatids in different stages of the spermatogenic cycle (Fig. 2). At stage V, elongated spermatids are localized mainly in the middle part of the tubule, corresponding with the D2-positive cells (Fig. 2A and B). A dense D2 labeling coincided with the localization of more mature elongated spermatids close to the tubule lumen (Stage VII/VIII; Fig. 2C and D).

In contrast to the dense deiodinase labeling on spermatids, other spermatogenic cells, such as spermatogonia and spermatocytes, were virtually negative. Of note, interstitial cells (Fig. 1C) were negative for the presence of D2 mRNA.

D2 activity in separated testicular cell fractions and other tissues

To provide additional evidence for D2 localization in germ cells, we next evaluated the enzyme activity in two separated testicular somatic and germinative enriched cell fractions of both euthyroid and hypothyroid groups. D2 activity was mainly detected in the germ cell fraction from testes of euthyroid rats (0.23 ± 0.003 vs 0.02 ± 0.013 fmol/min per mg protein), further confirming the D2 expression profile obtained by real-time PCR and *in situ* hybridization. The 5' deiodination activity in isolated germ cells was then characterized by kinetic studies that showed a typical K_m (T_4) for D2 (1.1 nM) and V_{max} of 0.63 fmol T_4 /min per mg protein (Fig. 3A). D2 activity was completely abolished by iopanoic acid (100 μ M) and higher T_4 concentration (100 nM). Hypothyroidism induced a significant increase in enzyme activity (approximately twofold).

D2 expression was also evaluated in other tissues of the adult reproductive system. D2 activity was readily detected in

Figure 2 Comparison of the positions of spermatids and D2 labeling within the seminiferous epithelium of rats by high-resolution light microscopy (HRLM; A and C) and *in situ* hybridization (B and D). A and B shows the same stage (V) of the cycle. Observe that the position of elongate spermatids (A, dark transparent areas) coincide with the position of D2 labeling in B. Other cells of the seminiferous epithelium, such as spermatogonia (G), pachytene (P), round spermatids (R), and Sertoli cells (S), distinguished in A, are apparently not labeled in B (asterisks). L, lumen. In C and D, at stage VII, the elongated spermatids are almost ready to be released and, in this way, they are concentrated and positioned as a palisade on the surface of the epithelium (C, dark transparent area). In D, the dense D2 labeling is localized to the same position where the elongated spermatids are seen and virtually absent in the rest of the seminiferous epithelium. T, high concentration of spermatid tails. Note that the difference between the number of spermatids in HRLM and the high concentration of D2 labeling by *in situ* hybridization is due to the difference of section thickness (1 and 14 μ m respectively). R5, step 5 round spermatid; R7, step 7 round spermatid; IC, interstitial cells. Bars represent $\sim 12 \mu$ m.

Figure 3 (A). D2 kinetics from testicular germ cell samples in adult rat. Representative Lineweaver-Burk plot of T_4 deiodination catalyzed by D2. Kinetic constants were calculated to be K_m 1.1 nM and V_{max} 0.63 fmol/min per mg protein. (B). D2 activity in rat reproductive organs. Data are representative of three experiments.

prostate in euthyroid rats and increased several fold in hypothyroid animals (0.03 vs 0.093 ± 0.014 fmol/min per mg protein respectively, $P < 0.001$). D2 activity was undetectable in epididymus, PBS-washed seminal vesicle, and spermatozoa (Fig. 3B).

Discussion

This study demonstrates for the first time that D2 expression is highly concentrated in a specific type of germ cells in the seminiferous epithelium of adult rat testis. By *in situ* hybridization histochemistry, using a probe complementary to the coding sequence of rD2mRNA, we have shown that *Dio2* transcripts are localized almost exclusively in spermatids undergoing maturation. D2 mRNA and activity measured in isolated somatic and germinative cell fractions confirmed the hybridization results. In the other tissues of male reproductive tract evaluated, D2 expression was only detected in the prostate. Altogether, our results provide direct evidence for a specific localization and activity of D2 in elongated spermatids within the testis.

Thyroid hormones play an important role in rat testicular development, especially on differentiation of Sertoli cells and spermatogenesis in the pre-pubertal stage (Holsberger & Cooke 2005). Experimentally induced neonatal hypothyroidism has been shown to lengthen the proliferative period of Sertoli cells, resulting in a significant increase in adult testis size and sperm production (Hess *et al.* 1993). In contrast, experimental hyperthyroidism induces Sertoli cell differentiation (Jannini *et al.* 1993). Furthermore, as recently reported in the *rdw* rats with congenital hypothyroidism, thyroid hormone seems to be important for the maintenance of testicular structure after full maturation (Sakai *et al.* 2004). Although thyroid hormones are the best described modulators of the developing testis,

their role in the adult organ is still undefined (Holsberger & Cooke 2005). A critical development in this field was the finding that TRs are also present, at a low but significant level, in rat and human adult testis (Buzzard *et al.* 2000, Jannini *et al.* 2000), indicating that T_3 may have a direct effect on the adult organ.

We and others have previously shown that D2 is expressed in adult mice and rat testis, and that its activity is significantly induced by hypothyroidism (Bates *et al.* 1999, Wagner *et al.* 2003). Here, we further demonstrate that D2 expression is cell type-specific by real-time PCR analysis, *in situ* hybridization histochemistry, and enzymatic assays. Elongated spermatids at different stages of maturation appear to be the predominant germ cell positive for D2, whereas other germ cells, i.e. spermatogonia, spermatocytes, and round spermatids, are negative for D2.

In mammals, spermatogenesis is a complex process in which stem cells, the spermatogonia, go through a differentiation step followed by subsequent divisions and differentiation into mature spermatozoa (Russell *et al.* 1990). The spermiogenic phase is one of the most extraordinary cell transformations in the body. Despite numerous studies aimed at elucidating the hormonal control of spermatogenesis, there is still a debate as to which hormones are involved and at which sites they promote cell maturation and progression to the next stage of spermatogenic cycle (El Shennawy *et al.* 1998, Holdcraft & Braun 2004). High D2 activity is typically found in specialized tissues, such as the anterior pituitary, CN, and BAT, where T_3 concentration seems to be critical (Bianco *et al.* 2002). The finding that D2 is highly expressed in the elongating spermatid, at the middle of the spermiogenic process, suggests a role for T_3 in spermatid differentiation. It is important to note that TR $\alpha 1$, one of the functional TR isoforms, and thyroid hormone transporters known to facilitate cellular thyroid

hormone uptake have been identified in germ cells, not only in adult rats but also in humans (Buzzard *et al.* 2000, Jannini *et al.* 2000, Suzuki *et al.* 2003). Since the biological effects induced by thyroid hormones are mediated by specific nuclear receptors, it is possible that the intracellular D2-generated T₃ may induce spermatids differentiation playing a role in their maturation process.

Previous studies have shown that hypothyroidism in post-pubertal period has little effect on testis, and fertility is seldom compromised (Maia *et al.* 1990, Jannini *et al.* 1995, Schneider *et al.* 2006). A potential explanation for these observations is that increased D2 activity, induced by the hypothyroidism, maintains T₃ concentration at normal levels, thus preserving the spermiogenic process and fertility from this adverse effect. Interestingly, 5' deiodination activity was also reported in seminal plasma of pre-pubertal pigs (Brzezinska-Slebodzinska *et al.* 2000).

Based on the findings of this study, it is somewhat intriguing that mice lacking the Dio2 (*Dio2*^{-/-}) gene appear to have normal reproductive function (St Germain *et al.* 2005). The most likely explanation is that the normal circulating thyroid hormone levels observed in these animals are adequate to allow tissue development and function (de Jesus *et al.* 2001).

In conclusion, we have shown that D2 expression is highly concentrated in elongated spermatids, whereas other germ cells and Sertoli cells of the seminiferous epithelium are virtually negative for this enzyme. These results suggest that thyroid hormone might have an effect on spermatogenesis in the adult rat testis, specifically on the spermiogenic or differentiation phase.

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