Orchidectomy of middle-aged rats decreases liver deiodinase 1 and pituitary deiodinase 2 activity

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Abstract

Endogenous androgens are involved in regulation of thyroid function and metabolism of thyroid hormones. As serum testosterone level progressively declines with age, this regulation may change. We tested how androgen deprivation, achieved by orchidectomy, affects thyroid homeostasis in middle-aged rats. Fifteen-month-old Wistar rats were orchidectomized (Orx) or sham-operated under ketamine anesthesia (15 mg/kg body weight). Five weeks after the surgery, animals were decapitated. Thyroids were used for histomorphometric and ultrastructural examinations and together with livers and pituitaries for real-time quantitative PCR and deiodinase (DIO) activity measurements. Serum testosterone, TSH, L-thyroxine (T₄), and cholesterol (Chol) levels were determined. As expected, middle-aged control rats had lower ($P < 0.05$) testosterone and T₄ compared with 3-month-old males. In the Orx middle-aged group, we

detected diminished serum testosterone ($P < 0.05$), no change in TSH and T₄ levels, and higher Chol level ($P < 0.05$), in comparison with age-matched controls. Histomorphometric analysis of thyroid tissue revealed decreased relative volume densities of follicles and colloid ($P < 0.05$). Relevant gene expressions and DIO1 enzyme activity were not changed in the thyroids of Orx rats. Liver *Dio1* gene expression and DIO1 activity were decreased ($P < 0.05$) in comparison with the control values. Pituitary levels of *TSHβ*, *Dio1*, and *Dio2* mRNAs did not change, while DIO2 activity decreased ($P < 0.05$). In conclusion, orchidectomy of middle-aged rats affected thyroid structure with no effect on serum T₄ and TSH. However, decreased liver DIO1 and pituitary DIO2 enzyme activities indicate compensatory-adaptive changes in local T₃ production.

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Introduction

Homeostasis of thyroid hormone (TH) status is regulated by systemic control of TH release from the thyroid and by local enzymatic activation and inactivation of TH in extrathyroidal tissues that is catalyzed by iodothyronine deiodinase (DIO) enzymes. Endogenous sex steroids, both estrogen and testosterone, play an important yet poorly defined role in regulation of the hypothalamus–pituitary–thyroid (HPT) axis and TH homeostasis.

Clinical evidence demonstrated that female patients have higher incidence of thyroid dysfunction and carcinomas than men (Ron *et al.* 1987). However, elderly men with thyroid carcinomas have much worse prognosis than women (Morganti *et al.* 2005). In animal models, rats in particular, sexual dimorphism is more clearly evident than in humans: young adult males have higher serum TSH levels than females (Chen 1984, Banu *et al.* 2001, Marassi *et al.* 2007). In addition to effects on hypothalamic–pituitary hormone regulation (Donda *et al.* 1990, Köhrle *et al.* 1995, Schomburg & Bauer

1997), both testosterone and estrogen may differently affect thyroid homeostasis *in vivo* by affecting thyroid follicular epithelium (Pelletier & El-Alfy 2000, Banu *et al.* 2002), level of TH carrier proteins in circulation (Bisschop *et al.* 2006), and/or DIO enzyme activities in peripheral organs (Köhrle *et al.* 1995, Lisbôa *et al.* 2001).

Iodothyronine DIO plays an important role in maintaining systemic TH status, as well as local control of TH action. Three DIO isoenzymes have been identified in mammals (Bianco *et al.* 2002) so far: type 1 (DIO1), type 2 (DIO2), and type 3 (DIO3). Their expression and activity are highly development and tissue specific and depend on numerous hormonal, environmental, and nutritional factors (Köhrle 2002). DIO1 enzyme activity is mainly regulated by triiodothyronine (T₃; Köhrle 1994), but sex steroids may also be involved in this regulation. Liver DIO1 activity is higher in young male than in female rats (Harris *et al.* 1979, Donda *et al.* 1990, Miyashita *et al.* 1995), while pituitary DIO1 activity is lower in young males than in females (Köhrle *et al.* 1995, Lisbôa *et al.* 2001). It seems that sexual dimorphism in

this enzyme expression and activity may change with advancing age, as was reported for DIO1 activity in mouse liver (Schomburg *et al.* 2007).

Hormone level changes are an important part of aging. Aside from well-documented partial decline in circulating testosterone, aging could not be clearly associated with hypothyroidism in men (Lamberts *et al.* 1997). However, more recent studies suggest that elderly people and centenarians have higher serum TSH concentrations than younger individuals (Surks & Hollowell 2007, Atzmon *et al.* 2009). In male rats, aging is characterized by lower serum testosterone and TH levels, along with unchanged serum and pituitary TSH levels (Reymond *et al.* 1992, Cizza *et al.* 1995). At the level of hypothalamic–pituitary hormone regulation, aging seems to be associated with mild central hypothyroidism in both genders (Donda *et al.* 1990, Cizza *et al.* 1995).

Although numerous experimental studies have been undertaken to determine mechanisms and molecular targets of testosterone impact on thyroid homeostasis, results obtained so far are incomplete. Much more data are currently available for young than for aged animal models. The aim of our study was to determine changes in thyroid homeostasis after testosterone deprivation achieved by bilateral castration of middle-aged rats, which we used as a model of andropause. Besides hormonal, histomorphometric, and ultrastructural assessment of thyroid tissue, we examined changes in Dio gene expressions and activities, as potential molecular targets of testosterone action in the pituitary, thyroid, and liver.

Materials and Methods

Experimental animals

Male Wistar rats were housed in the unit for experimental animals at the Institute for Biological Research ‘Siniša Stanković’ in Belgrade, Serbia. They were kept individually under constant laboratory conditions – room temperature ($22 \pm 2^\circ\text{C}$) and lighting (12 h light:12 h darkness).

At the age of 15–16 months, animals were randomly divided into two groups: one was bilaterally orchidectomized (Orx, $n=12$) under ketamine anesthesia (15 mg/kg body weight) via scrotal route. The second group (SO; $n=12$) was sham-operated (SO), in which testicles were exposed but not removed. Three-month-old young adult rats (C; $n=6$) served as control group for SO middle-aged rats and were SO as well. Five weeks after the surgery, all animals were decapitated. Blood was collected from the trunk of both young and middle-aged animals and the sera were stored at -70°C .

The pituitaries, thyroids, and livers from middle-aged rats were excised and weighed. Organs from six animals were used for histological and ultrastructural examinations, while organs from another group of six animals were used for molecular and enzyme analyses.

The protocols were approved by the Animal Care and Use Committee of our Institute, following recommendations

provided in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123, Appendix A).

Determination of serum hormone and cholesterol levels

Total testosterone level was determined using enzyme immunoassay kit (Adaltis Italia spa, Bologna, Italy). According to the manufacturer, the lower limit of detection was 0.2 ng/ml, and the calibration range was up to 16 ng/ml. The sensitivity was 0.01 ng/ml. The cross-reactivity with DHT was 10%, with androstenedione 0.8%, androsterone 0.0%, and with DHEA-S 0.0%. All samples were assayed in duplicate together in one run, with intra-assay CV of 3.9%.

Serum TSH was measured with a commercially available rat TSH RIA kit (Immunodiagnosics Systems GmbH, Frankfurt am Main, Germany). According to the manufacturer, the lower limit of detection was 1.03 ng/ml and the calibration range was up to 30 ng/ml. The sensitivity was 0.33 ng/ml. The cross-reactivity with GH was $<0.1\%$, with Prl $<0.1\%$, with LH 5.1%, and FSH 0.4%. All samples were assayed in duplicate together in one run, with an intra-assay CV of 6.1%.

As rat serum has a different composition of TH binding proteins, we adapted human total thyroxine (T_4) RIA assay (INEP, Zemun, Serbia) by dissolving the standards in rat TH-free serum (Stringer & Wynford-Thomas 1982). The rat TH-free serum was depleted of endogenous iodothyronines by pretreatment with 8-anilino-1-naphthalenesulfonic acid followed by overnight charcoal adsorption. The calibration range of the novel RIA was from 12.5 to 500 nmol/l, with the lower limit of detection of 6 nmol/l. The intra-assay CV was 4.7–10%. The assay showed good median recoveries of about 108% (range 94.4–115.8%). No significant cross-reaction was observed with T_3 or with reverse T_3 (rT_3).

Total serum cholesterol (Chol) was determined with colorimetric CHOD/PAP assay, according to the manufacturer's instructions (Serbolab, Kragujevac, Serbia). According to the manufacturer, the limit of detection was 0–15.6 mmol/l. All samples were assayed in duplicate, with CV of 2%.

Histological, morphometric, and ultrastructural analyses

Thyroid glands were excised together with trachea. One thyroid lobe ($n=6$) was randomly chosen and further processed according to routine protocols for light microscopy. It was fixed in Bouin's fixative for 48 h and dehydrated in increasing concentrations of ethanol and xylene. After embedding in Histowax (Histolab Product Ab, Göteborg, Sweden), each tissue block was serially sectioned at 5 μm thickness on a rotary microtome (RM 2125RT Leica Microsystems, Wetzlar, Germany). Sections were further stained with hematoxylin and eosin (HE), according to standard procedures. Morphometric analyses of thyroids were carried out on HE sections, exactly as previously reported (Sosić-Jurjević *et al.* 2010). In brief, four to five transversal

sections from the anterior, central, and posterior parts of each animals thyroid were analyzed by point-counting method (Weibel 1979), using the M₄₂ multipurpose test grid inserted into the ocular of a Zeiss light microscope (Jena, Germany). Fifty test fields were counted per animal at a total magnification of $\times 400$.

Digital light microscopic images of thyroid tissue were made on a Leica DM RB Photo Microscope with a JVCTK 1280E Video Camera using the Qwin program (Leica) for the acquisition and analysis of the images.

For electron microscopy, sections of the other thyroid lobe ($n=4$) were immersed immediately in 4% glutaraldehyde in 0.1 mol/l PBS (pH 7.4) for 24 h and postfixed in 1% OsO₄ in the same buffer for 1 h. Tissue samples were dehydrated in a graded alcohol series and embedded in Araldite. Ultrathin sections were cut on LKB Ultramicrotome III (type 8802A; Sweden), using diamond knife (Diatome ultra 45°; Diatome, Switzerland), stained with uranyl acetate and lead citrate, and examined under a MORGAGNI 268 (FEI Company, Hillsboro, OR, USA) transmission electron microscope.

Sample collection and preparation of tissue homogenates

Pituitaries, thyroids, and livers were removed, immediately frozen in liquid nitrogen, and stored at -80°C for further processing. Frozen liver tissues ($n=6$) were first pulverized under dry ice (solid CO₂) in precooled Teflon containers, using a micro-dismembrator (B. Braun Biotech International GmbH, Melsungen, Germany). Aliquots of the powdered tissues were stored for further processing at -80°C .

RNA extraction and quantification

Pituitaries (for SO $n=6$; for Orx $n=4$) were initially homogenized with a TissueLyzer (Qiagen) and thyroids (for SO $n=6$; for Orx $n=6$) with Ultra Turrax (IKA Analytical, Staufen, Germany), and total RNAs were isolated using Qiagen RNeasy Mini kit (Qiagen). Total RNA from the liver tissue powder (for SO $n=6$; for Orx $n=6$) was isolated using the Trizol reagent (Invitrogen), according to the manufacturer's protocols. The purity and integrity of the total RNA of all samples were assessed using NanoDrop u.v.-vis spectrophotometer ND-1000 (PEQLAB, Erlangen, Germany). Absorbance ratio at 260/280 was calculated and found to be in the range of 1.8–2.0 for all RNA samples.

cDNA from all tissue homogenates was generated from 500 ng total RNA, using the iScript cDNA synthesis kit (Bio-Rad Laboratories). Transcript concentrations of *Dio1* (pituitary, thyroid and liver), *Dio2* (pituitary), *Spot14* (*Thsp*) (liver), hypoxanthine guanine phosphoribosyl transferase (*Hprt*; pituitary, thyroid, and liver), thyroid peroxidase (*Tpo*; thyroid), and thyroglobulin (*Tg*; thyroid) were determined by quantitative real-time PCR (qRT-PCR) analysis. In short, primers were chosen with the help of Primer3 (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). The primer sequences were

Dio1–f: 5'-TTTAAGAACAACGTGGACATCAGG-3'; *Dio1*–r: 5'-GGTTTACCCTTGTAGCAGATCCT-3'; *Dio2*–f: 5'-GCGACCTGACCACC TTTTACTAG-3'; *Dio2*–r: 5'-GCAGCACATCGGTCCTCTTG-3'; *TSH β* –f: 5'-TACAGAGACTTCACCTACAGAACG-3'; *TSH β* –r: 5'-GGCAACGGGGTAGGAGAAATAAA-3'; *Hprt*–f: 5'-TTATGGACAGGACTGAAAGACTTG-3'; *Hprt*–r: 5'-CAGCAGGTCAGCAAAGAACTTATA-3'; *Tpo*–f: 5'-TTGGATCTGGCATCACTGAAGTT-3'; *Tpo*–r: 5'-ATCTTGTGACCATGCTTCTGTTG-3'; *Tg*–f: 5'-CCGG-ATATTGCAGAGACGAT-3'; *Tg*–r: 5'-GGCAAGCTTG GGATATATGGA-3'; *Spot14*–f: 5'-CTTACCCACCTGACCCAGAA-3'; *Spot14*–r: 5'-CATCGTCTTCCCTCTCGTGT-3'. Amplifications were conducted in duplicate with the Absolute qPCR SYBR Green Fluorescein Mix (Thermo Fisher Scientific, Inc., ABgene products, Epsom, UK), on a RT-PCR detection system (iCycler; Bio-Rad Laboratories). The program included the following steps: initial denaturation at 95°C for 10 min, followed by 40 cycles, each including a denaturation step at 95°C for 30 s, an annealing step at 60°C for 45 s, and an elongation step at 72°C for 30 s. At the end of a final elongation step at 72°C for 3 min the reaction mix was cooled at 8°C .

The optimization of PCR and validation of all PCR primer sets used in this study was previously determined (<http://edoc.hu-berlin.de/docviews/abstract.php?id=36909>). *Hprt*, *Gapdh*, and *18S rRNA* were found to be most stably expressed genes in different rat tissues under different experimental conditions (methimazole or T₄ treatment, gonadectomy, and subsequent hormone replacement). Based on these data, in this study, the same three genes were also used as internal controls for three replicate assays with each primer set. Similar results were obtained in terms of statistical significance for all reference genes, but *Hprt* had the most similar expression level and amplification efficiency with all examined genes in different tissues.

Figure 1 Serum total testosterone (a), TSH (b), total thyroxine (T₄; c), and cholesterol (Chol; d) levels of control young adult (C), sham-operated (SO) and orchidectomized (Orx) middle-aged rats; serum testosterone was determined by enzyme immunoassay, while TSH and T₄ were determined by RIAs. Statistics: one-way ANOVAs and Bonferroni's multiple range *post hoc* test. The values are mean $\pm$ s.d.; $n=5-7$; ^a $P<0.05$ compared with C rats; ^b $P<0.05$ compared with SO rats.

Figure 2 Thyroid gland of control sham-operated (a) and orchidectomized (b) middle-aged rat. Light microscopy, hematoxylin eosin staining.

Amplification efficiency was determined from the slopes of dilution curves and the efficiency range for all examined genes (and all examined organs) was between 84 and 107%. Specificity of the amplification product for each primer set was verified by analytical gel electrophoresis and by melting curve analyses. None of all primer sets showed dimers in melting curves or on agarose gels. Diethylpyrocarbonate (DEPC) H₂O was used as negative control instead of cDNA template.

Protein extractions and 5'-iodothyronine DIO activity measurements

Pituitary (~15 mg; *n*=6) and liver tissue powder aliquots (~50 mg; *n*=6) were sonicated in 300 and 500 µl of the homogenization buffer, pH 7, respectively, on ice. The buffer

contained 250 mmol/l D(+)-sucrose (Carl Roth, Karlsruhe, Germany), 20 mmol/l 4-(2-hydroxyethyl)-1-HEPES (Merck, and 0.5 mmol/l EDTA (Sigma-Aldrich), pH 8.0, and 1 mmol/l dithiothreitol (DTT; Sigma-Aldrich). After sonication (2×10 pulses, 0.6 s at 200 W; B. Braun Biotech), the membrane fraction of liver proteins was separated by centrifugation (4 °C, 10 000 *g*, 10 min). The pellet was resuspended in 400 µl of homogenization buffer and sonicated again.

For protein extraction from the thyroids, one thyroid lobe (~14 mg; *n*=6) was homogenized in 300 µl of the same homogenization buffer on ice, using the Ultra Turrax (IKA Analytical). The homogenates were further centrifuged at 10 000 *g* for 20 min. Supernatants were removed and pellets were resuspended in homogenization buffer. Protein concentrations were determined by a modified Bradford assay using IgG as standard (Bio-Rad).

DIO activities in pituitary, thyroid, and liver were measured based on previously described methods (Baur *et al.* 2000, Schomburg *et al.* 2006). In brief, the protein samples were assayed in a reaction mixture containing 60 µg (pituitary), 7.5 µg (thyroid), or 20 µg (liver) proteins. DTT (10 mmol/l) served as cofactor and [¹²⁵I]rT₃ was added as tracer (~30 000–80 000 c.p.m./tube, specific activity: 25 TBq/mmol, PerkinElmer, Rodgau-Jügesheim, Germany). The substrate concentration was 2 nmol/l rT₃ for pituitary or 1.0 µmol/l rT₃ for both thyroid and liver. The reaction volume was 300 µl; the reaction proceeded at 37 °C for 2 h (pituitary and thyroid) or 1 h (liver). Determinations of DIO1 activity were performed in sextuplicate (half in the presence and the other half in the absence of 6-*n*-propyl-2-thio-uracil, PTU). Conditions had been optimized so that deiodination of the substrate was <15%. For pituitaries and thyroids, the fraction of iodide release blocked by 1 mmol/l PTU was ascribed to DIO1. However, no thyroid DIO2 activity has been detected. Before use, [¹²⁵I]rT₃ was purified from free (¹²⁵Iodide) by chromatography using Sephadex LH-20 (Sigma-Aldrich).

After starting the enzymatic reaction by adding [¹²⁵I]rT₃-containing substrate mix, the samples were incubated in a water bath at 37 °C. The reaction was stopped by adding 50 µl BSA-PTU stop solutions and 10% trichloroacetic acid for precipitation of protein. After centrifugation (20 °C, 14 000 *g*, 5 min), the supernatant was transferred on Dowex-50 WX-2-column (Bio-Rad

Table 1 Morphometric parameters of the thyroid gland in sham-operated (SO) and orchidectomized (Orx) middle-aged rats. The results were obtained on four to five transversal sections from the anterior, central, and posterior parts of the thyroid, using M₄₂ test system (50 test fields per animal) and further analyzed by an unpaired Student's *t*-test. The values are mean±s.d. (*n*=6)

Group	τ (µm)	ia=V _{ve} /V _{vc}	V _{ve} (%)	V _{vi} (%)	V _{vc} (%)	V _{vf} (%)
SO	9.9±0.3	1.4±0.1	38.0±2.4	32.5±3.9	29.5±2.9	67.6±2
Orx	10.4±0.5	1.5±0.2	37.8±1.4	36.7±1.6*	25.4±1.9*	63.8±0.7*

τ, the height of thyroid follicular cells; ia, index of activation rate; V_{ve}, relative volume densities of epithelium; V_{vi}, interstitium; V_{vc}, colloid; V_{vf}, follicles. **P*<0.05 vs SO rats.

Laboratories). Released ^{125}I iodide was eluted by adding 10% acetic acid. ^{125}I -Iodide samples were measured on a gamma counter (Gamma Master, LKB Wallac, Turku, Finland) and background corrected. The mouse liver homogenate of known protein concentration was used as interassay variability control. After the assay, protein concentration from all samples was again determined by the Bradford assay, and the obtained results were used for calculation of the enzyme activities as described previously (Schomburg *et al.* 2006).

Statistical analysis

The statistical analyses were performed using GraphPad Prism v.5.01 for Windows (GraphPad Software, San Diego, CA, USA). The data are expressed as mean $\pm$ S.D., except for qRT-PCR results, which represent mean $\pm$ S.E.M. of the fold changes compared with basal levels of *Hprt* as reference gene.

One-way ANOVA was used to compare results on serum hormone and Chol levels, followed by the *post hoc* Bonferroni's multiple range tests to detect differences between groups. The differences in morphometric results, DIO activities and organ weights between groups were evaluated by the unpaired Student's *t*-test. The paired *t*-test was used to analyze the changes in body weight between SO and Orx groups. qRT-PCR data were calculated and evaluated using the Relative Expression Software Tool-Multiple Condition Solver (REST-MCS, version 2), as described (Pfaffl 2001). The minimum level of significance was set at $P < 0.05$.

Results

Body and organ weights

The mean body weight of SO animals before the experiment was 675 ± 72 g and did not significantly change by the end of the experiment (691 ± 59 g). However, in the Orx group, significant body weight loss was revealed (by 11%, $P < 0.05$). For the SO group, the mean pituitary, thyroid, and liver weights were 15 ± 1.9 mg, 33 ± 3 mg, and 17.4 ± 1.9 g respectively. No significant changes in organ weights were detected in the Orx group (the mean pituitary, thyroid, and liver weights were 16.3 ± 2.9 mg, 28.6 ± 3.5 mg, and 15.4 ± 2.1 g respectively).

Serum hormone and Chol levels

Middle-aged rats had 50 and 34% ($P < 0.05$) lower serum total testosterone and T_4 respectively in comparison to young male adults. No age-related changes in serum TSH level were detected (Fig. 1).

Figure 3 Thyroid follicular cells (FC) from sham-operated (a and b) and orchidectomized (c and d) middle-aged rat. BC, red blood cells; BV, blood vessel; CC, thyroid C cell; N, nucleus; RER, rough endoplasmic reticulum; LY, lysosomes; DB, dense bodies; C, colloid; AM, apical membrane; CR, crystalline inclusions. Transmission electron microscopy.

Surgical removal of both testicles in middle-aged rats resulted in reduced total serum testosterone by 90% ($P < 0.05$) compared with values obtained for age-matched SO controls. No change in serum concentration of TSH and total T_4 was detected. Serum total Chol was 25% higher ($P < 0.05$) compared with the SO rats (Fig. 1).

Thyroid gland: histomorphometric and ultrastructural findings

Thyroid parenchyma of control SO middle-aged male rat consists of follicles of different sizes. Still, follicles composed of cuboidal follicular epithelium and moderate portion of dense luminal colloid were predominant (Fig. 2a). In Orx rat, thyroid follicles displayed a higher variation in size, and follicles of smaller size appeared more frequent in comparison with the SO rats (Fig. 2b). Further morphometric analyses demonstrated decreased relative volume of the follicles and colloid, by 15 and 17% ($P < 0.05$) respectively, accompanied by a 12% ($P < 0.05$) increase in relative volume density of interstitium in Orx rats (Table 1).

Ultrastructurally, thyrocytes of middle-aged SO rats had a cuboidal shape and polar organization of organelles – oval nuclei were at the base of the cells, followed by well-developed rough endoplasmic reticulum (RER), lysosomes, dense bodies, and microvilli projected into the colloid at the apical surface (Fig. 3a). However, quite a few thyrocytes were characterized by dilated and disoriented RER, without losing their cuboidal shape. Some of these contained geometrically shaped (rhomboid or rectangle) crystalline inclusions, encircled by proliferated and dilated RER (Fig. 3a and b). In thyroids of Orx rats, similar ultrastructural organization of thyrocytes was observed, though no crystalline inclusions were detected (Fig. 3c and d).

Expression of genes relevant to the HPT axis in the pituitary, thyroid, and liver

In the pituitary, gene expressions of *Dio1* and *Dio2* and TSH-specific β subunit were examined. Upon quantification, no significant difference in mRNA levels of these genes was detected between SO and Orx groups (Fig. 4). In the thyroid, mRNA levels of *Dio1*, *Dio2*, *Tg*, and *TPO* were not significantly different between the Orx middle-aged group and SO rats (Fig. 5).

In the liver of Orx group, *Dio1* expression was 2.7-fold lower ($P < 0.05$) than the value obtained for the SO group (Fig. 6a). Expression of the *Spot14* gene did not significantly differ (Fig. 6b).

DIO activities in the pituitary, thyroid, and liver

As shown in Fig. 7 orchidectomy of middle-aged male rats induced a significant decrease in pituitary DIO2 and liver DIO1 activities, by 27 and 33% respectively ($P < 0.05$). Pituitary and thyroid DIO1 activities were unchanged in comparison to the SO controls.

Figure 4 Gene expression of type 1 (*Dio1*; a) and 2 (*Dio2*; b) iodothyronine deiodinases and *TSHβ* (c) in the pituitary of sham-operated (SO; $n = 6$) and orchidectomized (Orx; $n = 4$) middle-aged rats. The relative gene expression analyses were performed by quantitative real-time PCR method. Statistics: Relative Expression Software Tool-Multiple Condition Solver, the values are mean $\pm$ S.E.M. of the fold changes compared with basal levels of *Hprt* as reference gene.

Discussion

This study demonstrates that castration of middle-aged rats induces small changes in thyroid structure, namely reduction in relative volume of follicles, accompanied by the corresponding changes of some components of the HPT axis, such as reduced activities of DIO1 in the liver and DIO2 in the pituitary. Serum concentrations of TSH and TH remained unchanged.

In this experiment, orchidectomy was applied as a classical approach for studying the effects of endogenous androgens on hormonal homeostasis. As expected, orchidectomy diminished serum testosterone levels (by 90%) in our model of andropause. A small but significant decrease in body weight was detected in the Orx group, which may be due to a skeletal muscles atrophy (Rincon *et al.* 1996, Axell *et al.* 2006) and/or decreased food intake (Gentry & Wade 1976) induced by lack of androgens. Serum testosterone level in control middle-aged males was less than half that in young adults. Other researchers also demonstrated decreases in blood testosterone concentration with advancing age (Chen *et al.* 1994, 1996). Age-related partial decline of serum testosterone in men is associated with decline in sexual function, muscle function, bone density, and other physiological parameters, popularly termed andropause (Harman *et al.* 2001, Matsumoto 2002).

In addition to the decline in serum testosterone, in our middle-aged rats, T_4 levels were significantly decreased in comparison with young adult males. Thyroid tissue of SO rats was characterized by the presence of inactive follicles, distended with dense colloid. Rao-Rupanagudi *et al.* (1992) and other researchers (Cizza *et al.* 1992, Mariotti *et al.* 1995) also reported similar histological changes and progressive loss of follicular tissue with advancing age in the thyroid of male Sprague-Dawley or Fisher rats respectively with significant decreases in free serum TH levels.

Figure 5 Gene expression of type 1 (*Dio1*; a) and 2 (*Dio2*; b) iodothyronine deiodinases, thyroglobulin (*Tg*; c), and thyroid peroxidase (*Tpo*; d) in the thyroid of sham-operated (SO; $n=6$) and orchidectomized (Orx; $n=6$) middle-aged rats. The relative gene expression analyses were performed by quantitative real-time PCR method. Statistics: Relative Expression Software Tool-Multiple Condition Solver, the values are mean $\pm$ S.E.M. of the fold changes compared with basal levels of *Hprt* as reference gene.

At the ultrastructural level, both in SO and in Orx middle-aged rats, besides numerous follicular cells with typical organization of cell organelles, quite a few thyrocytes were characterized by dilated and distended RER cisternae. Similar ultrastructural changes were previously reported in thyrocytes of aged mouse thyroid (Tamura & Fujita 1981, Nève & Rondeaux 1991). Tamura & Fujita (1981) described such ultrastructural changes in thyrocytes of 'active' follicles as degenerative. Both groups related them to follicular cell hyperactivity explained with compensation for lack of TH production in inactive, so-called 'cold' mice follicles. Further quantitative ultrastructural studies with rats at different life stages are needed to understand the observed features of thyrocytes under our experimental conditions.

In the thyroids of Orx middle-aged rats, no significant changes were detected in expression of genes involved in TH synthesis (*Tg* and *Tpo* genes) and deiodination (*Dio1* and *Dio2* genes). Similar to results of our previous study (Šošić-Jurjević *et al.* 2010), morphometric analyses of thyroid sections revealed changes in thyroid structure, namely reduction in relative volume density of follicles and colloid. Taking all obtained results together, we suppose that these histomorphometric changes reflect increased colloid reabsorption in Orx middle-aged rats.

Apart from the hormone synthesis and secretion by the thyroid gland, deiodination pathways in liver and kidney are the main contributors to TH metabolism, turnover, and homeostasis (Schweizer *et al.* 2008). High levels of DIO1 activity were found in these peripheral organs, both in rats and in humans (Visser 1996). Under our experimental conditions, a decrease in liver *Dio1* expression, accompanied by a corresponding decrease in enzyme activity, was detected in Orx middle-aged rats, in line with other authors who demonstrated the same for Orx prepubertal and adult rats (Miyashita *et al.* 1995, Lisboa *et al.* 2001, Marassi *et al.* 2007).

As no change in serum TH hormones was detected in Orx middle-aged males, this effect may be attributed to testosterone deprivation. In accordance with this, Miyashita *et al.* (1995) demonstrated that liver DIO1 activity in young adult males was higher than in females, both in euthyroid and thyroidectomized animals.

Differential effects of sex hormones on *Dio1* expression in liver and kidney were reported to change with advancing age. Thus, sexual dimorphic expression of liver *Dio1* that was detected in young mice vanished at the age of 1 year, while sex-specific differences in renal *Dio1* mRNA expression sustained with increasing age (Schomburg *et al.* 2007). Contrary to this, our results in middle-aged rats demonstrate preserved responsiveness of liver *Dio1* to testosterone deprivation, while *Dio1* expression in the rat kidney was not affected by castration (results not shown). Species-specific differences in age-related changes of *Dio1* expression profile in the peripheral organs may indicate species-specific changes in metabolism and degradation of TH in aged animals. Further studies and comparisons of different animal species at different life stages are needed to determine the factors contributing to these changes.

Our results clearly demonstrate that liver DIO1 significantly decreased in Orx middle-aged rats. Decreased local production of T_3 in the liver could act locally on expression of hepatic T_3 -responsive genes. However, we observed no change in expression of T_3 -regulated *Spot14* gene in Orx middle-aged rats. The responsiveness of this gene to T_3 seems to be decreased with advancing age (Mooradian *et al.* 1991). An increase in serum Chol level was detected in Orx middle-aged males, and we cannot rule out that a local decrease in T_3 level might contribute to this change. Further examinations of expression profiles of other hepatic T_3 -responsive genes involved in regulation of Chol metabolism are needed to evaluate the significance of Orx-induced decline in DIO1 enzyme activity to decline in hepatic T_3 .

We further investigated whether deprivation of endogenous androgens affects pituitary TSH synthesis and secretion and whether DIO1 and DIO2 enzymes play a role in this regulation. No significant change in *TSH β* gene expression and no change in serum TSH level were detected in Orx rats in comparison to the age-matched controls. However,

Figure 6 The expression levels of *Dio1* (a) and *Spot14* (b) genes in liver of sham-operated (SO; $n=6$) and orchidectomized (Orx; $n=6$) middle-aged rats; the relative gene expression analyses were performed by quantitative real-time PCR method. Statistics: Relative Expression Software Tool-Multiple Condition Solver, the values are mean $\pm$ S.E.M. of the fold changes compared with basal levels of *Hprt* as reference gene; * $P<0.05$ compared with SO rats.

Figure 7 Enzyme activities of type 1 (DIO1) and type 2 (DIO2) iodothyronine deiodinase in pituitary (a and b), thyroid (c), and liver (d) of sham-operated (SO; $n=6$) and orchidectomized (Orx; $n=6$) middle-aged rats; enzyme activities were determined using the radioactivity-based assay. The results were evaluated by unpaired Student's *t*-test. The values are mean $\pm$ S.D.; * $P<0.05$ compared with SO rats.

decreased DIO2 activity detected under our experimental conditions suggests some adaptation or compensation of local T_3 production to altered thyroid or androgen status sensed by pituitary.

It was previously documented that the negative feedback control of TSH production by the anterior pituitary involves DIO enzymes, which are distinctly regulated by THs: DIO1 activity is upregulated by T_3 (Köhrle 2002), while DIO2 is upregulated by low T_4 (Christoffolete *et al.* 2006). As no change in serum T_4 was detected in this experiment, decreased DIO2 activity may be attributed to lack of endogenous androgens in Orx rats.

In addition, young male rats were reported to have lower DIO1 activity in the pituitary than the females (Köhrle *et al.* 1995, Lisbôa *et al.* 2001). Besides, pituitary DIO1 activity was increased in castrated young male rats, though subsequent testosterone administration did not affect it (Lisbôa *et al.* 2001). Therefore, it was speculated that a difference in serum TSH between males and females is, at least, partly due to change in DIO1 enzyme activity. It is well known that castration of young adult males significantly reduces circulating levels of TSH (Christianson *et al.* 1981, Bagchi *et al.* 1984, Borges *et al.* 1998, Marassi *et al.* 2007). However, in line with the results of this study, those who examined the effects of castration in older males demonstrated no effect on TSH level in circulation (Bagchi *et al.* 1984, Chen 1984).

Our results, in the context of the results of other authors, implies that the role of endogenous androgens in pituitary TSH regulation diminishes with advancing age and that age-related changes in control of DIO enzyme expression and activity may contribute to this effect. Correa da Costa & Rosenthal (1996) reported a significantly decreased pituitary DIO2 activity in pooled pituitary samples from 10- to 12- and 24- to 30-month-old female rats in comparison with the young adult females, while the DIO1 activity only became

significant in the senescent rats. However, it is also possible that decreased local pituitary production of T_3 (due to decreased DIO2) under our experimental conditions was in part compensated by T_3 from the serum. Further examinations at different time points during adulthood or after prolonged Orx are needed to distinguish between these two hypotheses. In addition, androgen-modulated paracrine regulation between different pituitary cell types, which express distinct levels of DIO enzymes and/or TH transporters, might influence the fine tuning of anterior pituitary feedback regulation of the HPT axis with increasing age (Baur *et al.* 2002, Denef 2008, Liao *et al.* 2011).

In conclusion, our results demonstrate that orchidectomy of rats at middle age induced changes in thyroid structure with no effect on serum T_4 and TSH. However, decreased activities of DIO1 in the liver and DIO2 in the pituitary indicate tissue compensation or adaptation through changes in local production of T_3 .

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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