Pharmacological Doses of Testosterone Upregulated Androgen Receptor and 3-Beta-Hydroxysteroid Dehydrogenase/Delta-5-Delta-4 Isomerase and Impaired Leydig Cells Steroidogenesis in Adult Rats

Tatjana S. Kostic,2 Natasa J. Stojkov, Maja M. Bjelic, Aleksandar I. Mihajlovic, Marija M. Janjic, and Silvana A. Andric1,2

Reproductive Endocrinology and Signaling Group, Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, 21000 Novi Sad, Serbia

1To whom correspondence should be addressed at Reproductive Endocrinology and Signaling Group, Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Dositeja Obradovica Square 2, 21000 Novi Sad, Serbia. Fax: +(381) 21-450-620. E-mail: silvana.andric@dbe.uns.ac.rs.
2These authors contributed equally to this work.

Received January 11, 2011; accepted March 4, 2011

Anabolic androgenic steroids (AAS) are testosterone derivatives originally designed to enhance muscular mass and used for the treatment of many clinical conditions as well as in contraception. Despite popular interest and abuse, we still lack a broad understanding of effects of AAS on synthesis of steroid hormones on the molecular level. This study was designed to systematically analyze the effects of pharmacological/high doses of testosterone on steroidogenic machinery in Leydig cells. Two different experimental approaches were used: (1) In vivo experiment on groups of adult male rats treated with testosterone for 1 day, 2 weeks, and 2 months; (2) Direct in vitro testosterone treatment of Leydig cells isolated from intact rats. Result showed that prolonged in vivo treatment with testosterone decreased the expression of Scarb1 (scavenger receptor class B type 1), Tspo (translocator protein), Star (steroidogenic acute regulatory protein), Cyp11a1 (cholesterol side-chain cleavage enzyme), and Cyp17a1 (17a-hydroxylase/17, 20 lyase) in Leydig cells. Oppositely, the expression of Hsd3b (3-beta-hydroxysteroid dehydrogenase/delta-5-delta-4 isomerase), Ar (androgen receptor), and Pde4alb (cyclic adenosine monophosphate-dependent phosphodiesterases) was increased. Androgenization for 2 weeks inhibited Cyp19 (aromatase) transcription, whereas 2-month exposure caused the opposite effect. Direct in vitro testosterone treatment also decreased the expression of Cyp11a1, Cyp17a1, and Cyp19a1, whereas Hsd3b was upregulated. The results of expression analysis were supported by declined steroidogenic capacity and activity of Leydig cells, although conversion of pregnenolone to progesterone was stimulated. The upregulation of AR and 3βHSD in testosterone-impaired Leydig cells steroidogenesis could be the possible mechanism that maintain and prevent loss of steroidogenic function.

Key Words: testosterone; androgen receptor; 3βHSD; Leydig cell; steroidogenesis.

Testosterone and dihydrotestosterone (DHT) are critical androgenic steroid hormones that determine the expressions of the male phenotype, including the outward development of secondary sex characteristics as well as the initiation and maintenance of spermatogenesis (Kicman, 2008). Anabolic androgenic steroids (AAS), testosterone derivatives originally designed to enhance muscular mass, are used for the treatment of many clinical conditions and in contraception (Bagatell and Bremner, 1996; Bhasin, 2010; Bhasin et al. 2006; van Amsterdam et al., 2010). Potential health risks associated with nontherapeutic use of AAS (such as decreased sperm production, testicular atrophy, gynecomastia, increased chance of heart attacks, mood changes, liver cancer, premature stoppage of bone growth in younger males, etc.) by large numbers of healthy individuals are believed to be high (Basaria, 2010; Bhasin, 2010; Bhasin et al., 2006). Whereas historically misuse of AAS has been restricted to elite athletes, recent estimations indicate an increasing use by adolescents and rank the relative harm of AAS within a selection of 19 illicit drugs, including heroin, cocaine, ecstasy, and cannabis (van Amsterdam et al., 2010). Despite popular interest in AAS, we still lack a broad understanding of the effects of exposure to pharmacological/high doses of testosterone on biosynthesis of steroid hormones in vivo. Although human studies of these abused drugs are made difficult by their illicit nature, many of the AAS-induced changes should be recapitulated in animals, which therefore provide useful models for steroid abuse (Clark and Henderson, 2003). It has been shown that AAS dependence may pose a growing public health problem in future years but remains little studied, especially on the molecular level. More studies are needed to characterize AAS actions more clearly to improve treatment strategies (Kanayama et al., 2010).

Testosterone is secreted primarily by the Leydig cells of testes in males, and ovaries in females, although smaller amounts are produced by the adrenal gland in both sexes (Wang et al., 2009). Leydig cells of testes, like all other steroid-producing cells, synthesize steroid hormones from a common precursor, cholesterol. The specific requirement for cholesterol is met by...
selective uptake of extracellular cholesterol by receptors including the scavenger receptor type B1 (SCARB1). Transport of cholesterol from intracellular sources into the mitochondria is a rate-limiting and hormone-sensitive process that requires the presence of a specific complex of transport carrier proteins, including the translocator protein (TSPO) and the steroidogenic acute regulatory (StAR) protein (Stocco et al., 2005). Once delivered to the inner mitochondrial membrane, cholesterol is converted to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1, known as P450scct). Pregnenolone is further metabolized to progesterone by mitochondrial or microsomal 3β-hydroxysteroid dehydrogenases (3βHSD). In Leydig cells, maturation of progesterone to androstenedione is catalyzed by the 17α-hydroxylase/C17–20lyase (CYP17A1); although further conversion of androstenedione to testosterone is depend on activity of 17β-hydroxysteroid dehydrogenase (17βHSD), steroid dehydrogenase specific for androgen production. Testosterone can act directly on target cells through androgen receptor (AR) or it can be converted to DHT by the 5α-reductase or to estradiol by the aromatase (CYP19A1) (Payne and Hales, 2004). The steroidogenic function of Leydig cell is predominantly regulated by pituitary luteinizing hormone (LH) or its placental counterpart human chorionic gonadotropin (hCG), as well as in response to numerous steroidogenic stimuli, intratesticular factors, and signaling pathways. LH/hCG receptors activation leads to stimulation of adenylyl cyclase (Catt and Dufau, 1973; Dufau, 1998), accumulation in cyclic adenosine monophosphate (cAMP) intracellular levels, and the concomitant activation of the cAMP-dependent kinase (PRKA). The phosphodiesterases (PDEs) terminate cAMP signalling and have regulatory function in Leydig cells (Payne and Hales, 2004).

The lack of fundamental knowledge of the prolonged AAS actions on steroid-producing cells such as Leydig cells hampers our ability to understand the short- and long-term consequences of androgen use on particular elements of steroidogenic machinery in testosterone-producing Leydig cells. This study was designed to examine the effect of testosterone applied in vivo (for 1 day, 2 weeks, or 2 months) and in vitro (20 h) on components related to steroidogenic functions of Leydig cells. Doses used in the study are comparable with those used in clinical application or abused. We examined the expression of genes/proteins involved and/or related to steroidogenic synthesis and functions in Leydig cells.

MATERIALS AND METHODS

Materials

The antisera for StAR protein were generous gifts from Prof. Douglas Stocco (Clark et al., 1994), whereas the purified rabbit polyclonal antibody against 3βHSD was generous gifts from Prof. Ian Masson (Bain et al., 1991). Commercial materials and all other reagents are listed in detail and given as Supplementary material.

### TABLE 1

The Experimental Design of In Vivo Testosterone Application

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group abbreviation</th>
<th>Dose (per 100 g body weight)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T-1 day</td>
<td>0.5 mg</td>
<td>1-day treatment</td>
</tr>
<tr>
<td></td>
<td>T-2 weeks</td>
<td>0.5 mg</td>
<td>2-week treatment</td>
</tr>
<tr>
<td>II</td>
<td>T-0.5 mg</td>
<td>0.5 mg</td>
<td>2-month treatment</td>
</tr>
<tr>
<td></td>
<td>T-2.5 mg</td>
<td>2.5 mg</td>
<td></td>
</tr>
</tbody>
</table>

Note. All control groups were received appropriate amount (50 µl per 100 g body weight) of sterile olive oil.

Detailed methodology for the studies included in this article was previously reported (Andric et al., 2007; Andric et al., 2010a,b; Kostic et al., 2008, 2010) and given in Supplementary material but is also briefly outlined here.

Ethical Approval

All the experimental protocols were approved by the local Ethical Committee on Animal Care and Use at the University of Novi Sad operating under the rules of National Council for Animal Welfare and following statements of National Law for Animal Welfare (copyright March 2009). All our experiments were performed and conducted in accordance with the National Research Council (NRC) publication Guide for the Care and Use of Laboratory Animals (copyright 1996; National Academy of Sciences, Washington, DC) and NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80 23, revised 1996, 7th edition). All the experiments were carried out in the Laboratory for Reproductive Endocrinology and Signaling, Department of Biology and Ecology, Faculty of Sciences at University of Novi Sad.

Animals and In Vivo Treatment

Adult (3-month-old, 250–270 g body weight) male Wistar rats, bred and raised in the Animal Facility of the Faculty of Sciences (University of Novi Sad), were used for the experiments. The animals were raised in controlled environmental conditions (22 ± 2°C; 12 h light/dark cycle, lights on at 7 A.M.) with food and water ad libitum.

To mimic the most likely route of human exposure to androgens, we androgenized rats via weekly im injection of testosterone-enanthate (Testosteron-Depo, 250 mg/1 ml, 5 × 1 ml; ICN Galenica), the most extensively studied androgen for suppression of spermatogenesis (Anderson and Wu, 1996) and used in hypogonadal animals as replacement therapy (Morelli et al., 2004), as well as the most commonly used form of testosterone by both athletes and bodybuilders (Bhasin et al., 1996, 2001). Two types of experiments were performed (please see Table 1): (1) with 0.5 mg/100 g body weight of testosterone-enanthate during two time points (1 day T-1 day and 2 weeks T-2 weeks) and (2) with two doses of testosterone-enanthate (0.5 mg/100 g body weight or 2.5 mg/100 g body weight) during 2 months. In the first experiment, adult male rats were divided into three groups (each consist of six rats): (1) control group, (2) group treated with single im injection of testosterone-enanthate (0.5 mg/50 µl/100 g body weight) for 1 day, (3) group treated with a weekly im injection of same dose of testosterone-enanthate for 2 weeks (T-2 weeks). In the second type of experiment, we performed androgenization for 2 months but with two doses: one same as in the first type of experiments and the other one five times higher (2.5 mg/100 g body weight). Adult male rats were divided into three groups (each consist of six rats): (1) control group, (2) group treated with a weekly im injection of testosterone-enanthate (0.5 mg/50 µl/100 g body weight), and (3) group treated with a weekly im injection of testosterone-enanthate (2.5 mg/50 µl/100 g body weight). Because rats of all experimental groups...
were treated with intraperitoneal injections of testosterone-depot dissolved in olive oil, all rats in control groups received only 50 μl of olive oil per 100 g body weight. At the end of the administration period, all rats were quickly decapitated and trunk blood was collected. Individual serum samples were stored at −20°C until assayed for LH and androgens (T + DHT) levels, and isolation of Leydig cells from individual animals was performed. Experiments were repeated three times.

Preparation of Purified Leydig Cell and Ex Vivo Hormones Production

To follow ex vivo steroidogenesis, cAMP and elements of steroidogenic machinery (steroidogenic enzymes, proteins related to the steroidogenesis, and androgen/estrogen receptors), we used primary cultures of purified Leydig cells obtained from control and testosterone-treated rats prepared as described previously by our group (Andric et al., 2007, 2010a,b; Kostic et al., 2008, 2010). The proportion of Leydig cells present in culture was determined by staining for 3β HSD activity and was found to be 97.8 ± 1.3%. The viability was tested by using the 0.2% trypan blue dye exclusion test (Sigma Inc.) which determined total cell counts and ensured that greater than 90% of the cells were viable. The steroidogenic capacity of Leydig cells (estimated by dose-dependent stimulation with hCG) and the activity of steroidogenic enzymes (estimated by incubating cells with increasing concentrations of steroid substrates) were in line with those previously published (Andric et al., 2007, 2010a,b; Kostic et al., 2008, 2010). Purified Leydig cells obtained individually from six rats were pooled and plated in 90 mm Petri dishes (5 × 10^6 cells in 5 ml culture medium per dish). Three to five replicates of each pool/group were cultured and placed in to the CO2 incubator (34°C) for 3 h to attach and recover. At the end of recovery period, the culture medium was discarded and cells were incubated with M199-0.1% bovine serum albumin. After 2 h, cell-free media was collected and stored at −80°C prior to the measurement of progesterone, androgens, and estradiol levels, whereas glyceraldehyde monophosphate (cAMP) was measured in cell content. For experiments also measuring levels of transcripts (real-time PCR analysis) and proteins (Western blot), purified Leydig cell lysates were used as a source of RNA or protein. For ex vivo examination of the steroidogenic capacity of Leydig cells (estimated by dose-dependent stimulation with hCG) and the activity of steroidogenic enzymes (estimated by incubating cells with increasing concentrations of steroid substrates), Leydig cells obtained from control and androgenized rats were plated in 96-well plate (5 × 10^5 cells per well) in eight replicates of each pool/group and placed in to the CO2 incubator (34°C) for 3 h to attach and recover. At the end of recovery period, the culture medium was discarded and cells were challenged with increasing hCG-concentrations (0, 0.5, 5, and 50 ng/ml) to estimate steroidogenic capacity or with increasing concentrations (0, 0.1, 1, and 10μM) of different steroid substrates (pregnenolone, progesterone, and Δ4-androstenedione) to follow activity of steroidogenic enzymes. After 2 h, cell-free media was collected and stored at −80°C prior to the measurement of progesterone and androgens levels.

For in vitro experiments, primary cultures of purified Leydig cells (5 × 10^6 cells in 5 ml culture medium per dish; obtained from four normal rats) were incubated with increasing concentrations of testosterone (0.1, 1, 10, and 100μM and 1, 10, and 20μM) for 20 h. After 20 h, cells were washed and incubated for another 2 h in fresh medium. At the end of incubation, cells were used as a source of messenger RNA (mRNA) for real-time quantitative (RQ) PCR analysis.

Hormones and cAMP Measurement

Progesterone, testosterone, estradiol, and LH levels were measured by radioimmunoassay (Andric et al., 2007, 2010a,b; Kostic et al., 2008, 2010). Progesterone measurements were assayed in duplicate by radioimmunoassay (RIA) (sensitivity 6 pg per tube; intra-assay coefficient of variation 6.8%) using the anti-progesterone serum 337 (Andric et al., 2007; Kostic et al., 2008, 2010). Levels of androgens are referred to as testosterone + dihydrotestosterone (T + DHT) because the anti-testosterone serum 250 showed 100% cross-reactivity with DHT (Andric et al., 2007, 2010a,b; Kostic et al., 2008, 2010). All samples were measured in duplicate in one assay (sensitivity: 6 pg per tube; intra-assay coefficient of variation 5–8%). Estradiol levels in all samples were measured by RIA, also in duplicate in one assay, using the anti-estradiol serum 244 already described (Korenman et al., 1974). The limit of sensitivity was 5 pg, and intra-assay coefficient of variation was 7.7%. For serum LH levels, all samples were measured in duplicate, in one assay (sensitivity less than 1 ng/ml; intra-assay coefficient of variation 4.2%), by RIA according to the manufacturer’s protocol (ALPCO Diagnostic-LH (Rat) RIA), and the minimum detectable concentration has been assayed at 0.14 ng/ml (Andric et al., 2010b; Kostic et al., 2010). Level of cAMP in cell content of scraped purified Leydig cells was measured by the cAMP EIA Kit that permits cAMP measurement with a limit of quantification of 0.1 pmol/ml (at 80% B/B0) and IC50 of approximately 0.5 pmol/ml for acetylated cAMP samples (Andric et al., 2010b; Kostic et al., 2008).

RNA Isolation and complementary DNA Synthesis

Total RNA from purified rat Leydig cells were isolated using RNAeasy kit reagent following a protocol recommended by the manufacturer (www.qiagen.com). Following DNase-I treatment, first-strand complementary DNA was synthesized according to the manufacturer’s instructions (www.invitrogen.com). Negative controls consisting of nonreverse transcribed samples were included in each set of reactions. Quality of RNA and DNA integrity was checked by using primers for RS16 and GAPDH as described before by our group (Andric et al., 2007, 2010a,b; Kostic et al., 2008, 2010).

Real-Time PCR and Relative Quantification

The relative expression of the genes was quantitated by real-time PCR and two types of chemistries used to detect PCR products: SYBR Green-based and TLDA (TaqMan Low Density Array)-based detection.

SYBR Green. The relative expression of the genes by using SYBR Green for amplicon detection and ROX as an internal reference dye was done as previously described (Andric et al., 2010a,b; Kostic et al., 2010). Standard PCR settings were used in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) in presence of specific forward (F) and reverse (R) primers. The primers were design by using softver Primer Express 3.0 (Applied Biosystems) and full genes sequences from National Center for Biotechnology Information (NCBI) Entrez Nucleotide database (www.ncbi.nlm.nih.gov/sites/ entrez). The primers sequences used for real-time PCR analysis including GenBank accession codes for full genes sequences are given in Table 1 of Supplementary material and methods. Actin mRNA was also measured in the same samples and used to correct variations in RNA content among samples. A melt curve analysis was performed to ensure a single product was generated. The relative quantification of gene expression was calculated using ABI Prism 7900HT sequence detection system software and Relative Quantification Manager (Applied Biosystems). Relative quantification of each gene was done in duplicate, twice for each of three independent in vivo experiments (Andric et al., 2010a,b; Kostic et al., 2010).

TLDA rat phosphodiesterase panel assay.

The expression of the genes for PDEs in Leydig cells obtained from control and experimental rats were analyzed in real-time relative quantification by using the TLDA Rat Phosphodiesterase Panel Assay and ABI Prism 7900HT Sequence Detection System and Relative Quantification Manager Software as described previously by our group (Andric et al., 2010; Kostic et al., 2010). The Actb gene was used as an endogenous control and quantitated in the same real-time PCR as a part of TaqMan Low Density Rat Phosphodiesterase Panel and then used to correct for variations in RNA content among samples. Each sample was run in duplicate or triplicate, three times for each gene, for each of three independent in vivo experiments.

Protein Extraction and Western Blot Analysis

After incubation, Leydig cells (5 × 10^6 per well) were washed twice with ice-cold PBS, lysed, and Western blot analysis was performed as described previously by our group (Andric et al., 2007, 2010a,b; Kostic et al., 2008, 2010).
The immunodetection of the StAR protein was performed by using antisera against StAR protein generously supplied by Prof. Douglas Stocco (Clark et al., 1994), 3βHSD was detected with antibody kindly provided by Prof. Ian Masson (Bain et al., 1991), whereas AR was detected with polyclonal antibody (www.abcam.com). Actin was detected by using Actin detection kit (Oncogene Research Product). The reactive bands were always determined with a luminol-based kit, and the reaction was detected by an enhanced chemiluminescence system, using x-ray film. The immunoreactive bands were analyzed as two-dimensional images using the Image J (version 1.32; www.rsb.info.nih.gov/ij/download.html). The optical density (OD) of images is expressed as volume (OD³ area) adjusted for the background, which gives arbitrary units of adjusted volume (Andric et al., 2007, 2010a,b; Kostic et al., 2008, 2010).

**RESULTS**

**Effects of Androgenization for 1 Day and 2 Weeks**

In order to mimic the most likely route of human exposure to androgens, rats were androgenized via weekly im injection of testosterone with a clinical relevant dose of 0.5 mg/100 g body weight for 1 day (T-1 day) and 2 weeks (T-2 weeks).

**Testosterone Treatment for 1 Day or 2 Weeks Impaired Levels of Circulating LH/Testosterone and ex vivo Hormones/cAMP Production by Leydig Cells**

Im injections of testosterone for 1 day or 2 weeks caused significant decrease of LH (Fig. 1A) but increase level of androgens (Fig. 1B) in serum.

Steroidogenic activity of purified Leydig cells obtained from rats after in vivo treatment with testosterone was unchanged after 1-day treatment but significantly decreased after 2-week exposure to testosterone (Fig. 1). Progesterone productions remained unchanged (Fig. 1C), whereas production of...
androgens (Fig. 1D) and estradiol (Fig. 1E) was declined only after 2 weeks of testosterone treatment. In parallel, the production of cAMP also decreased (Fig. 1F).

Steroidogenic capacity of Leydig cells isolated from control and rats androgenized for 2 weeks was examined by ability of Leydig cells to respond in dose-dependent manner to hCG stimulation. Testosterone treatment decreased production of androgens (Fig. 1I), whereas progesterone production remained unchanged (Fig. 1G). Activity of steroidogenic enzymes in Leydig cells obtained from control and rats androgenized for 2 weeks was estimated by ability of Leydig cells to convert different steroidogenic substrates to progesterone and androgens. Conversion of pregnenolone to progesterone which reflects 3\(\beta\)HSD activity was significantly, dose-dependently, stimulated in Leydig cells from androgenized rats (Fig. 1H), whereas androgens production decreased (Fig. 1J). The conversion rate of progesterone to androgens declined in Leydig cells isolated from androgenized rats. In the presence of a saturating dose of progesterone, conversion rate was reduced about 50% (saturating dose) in Leydig cells from androgenized rat comparing with controls (Fig. 1K). The activity of 17\(\beta\)HSD measured by conversion of \(\Delta^1\)-androstenedione to testosterone dose-dependently declined in Leydig cells from rats treated with testosterone for 2 weeks (Fig. 1L).

Because testosterone treatment affected Leydig cells hormones/cAMP production, an expression analysis of elements involved and/or related with steroidogenic function of Leydig cells was performed.

**Testosterone Treatment for 1 Day or 2 Weeks Disturbed the Expression of Steroidogenic Machinery Elements in Leydig Cells**

Expression of specific components of steroidogenic machinery was analyzed by extracting mRNA from purified Leydig cells obtained from controls and androgenized male rats. Gene and protein expression levels were accessed by RQ-PCR and Western blot, respectively.

TLDA analysis showed that 2 weeks of testosterone treatment significantly enhanced expression of Pde4b, Pde7a, and Pde8b, whereas other PDEs responsible for cAMP degradation (Pde4a, Pde4c, Pde4d, Pde7b, and Pde8a) in adult rat Leydig cells remained unchanged (Fig. 2A). In the same samples, androgenization for 2 weeks stimulated the expression of dual-specific PDEs (Pde1c and Pde3a), whereas both types of the treatment, T-1 day and T-2 weeks, significantly increased the expression of PDE10a (Fig. 2B).

RQ-PCR analysis revealed that both time points of androgenization (T-1 day and T-2 weeks) significantly

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**FIG. 2.** Testosterone treatment for 1 day or 2 weeks disturbed the expression of steroidogenic machinery elements in Leydig cells. (A-C) Effects of testosterone on the quantitative gene expression of cAMP-specific (A), dual-specific (B) PDEs, and gene expression of elements involved in steroidogenesis of Leydig cells (C). (D) Effect of testosterone on AR, StAR, and 3\(\beta\)HSD protein expression in Leydig cells. In this and Figure 4B, a representative blot is shown in each panel, whereas pooled data from scanning densitometry normalized to actins values are shown as bars above the blots. Secretion activity of the Leydig cells used as a source of mRNA/protein is presented on the Figures 1C–1F. Data bars are group means ± SEM values of three independent in vivo experiments. Statistical significance between controls and testosterone-treated groups: *p < 0.05.
decreased the expression of CYP enzymes involved in androgenesis (Cyp11a1 and Cyp17a1), whereas Cyp19a1, responsible for aromatization and estrogen production, was decreased only with 2-week treatment with testosterone (Fig. 2C). Same type of treatment inhibited expression of Scarb1, Tspo, and Star. Oppositely, androgenization for 2 weeks stimulated Hsd3b and Ar expression (Fig. 2C). In the same time, no change was observed in the gene expression of Lhr, Sf1, Hsd17b, Shbg, and Esr1 (Fig. 2C).

Results of Western blot analysis were in line with the gene expression. Decrease in the level of StAR protein in Leydig cells obtained from rats androgenized for 2 weeks was observed (Fig. 2D). On the contrary, but to support gene expression, same treatment increased level of 3βHSD and AR protein expression in Leydig cells (Fig. 2D). Thus, elevated expression of 3βHSD, the key enzyme responsible for progesterone production, explained increased conversion of pregnenolone to progesterone in the same cells (Fig. 1C).

Effects of Androgenization for 2 Months

To study the potential impact of testosterone application for longer period, adult male rats were androgenized with two different doses of testosterone (0.5 mg/100 g body weight or 2.5 mg/100 g body weight) for 2 months.

Testosterone Treatment for 2 Months Impaired Levels of Circulating LH/Testosterone and Ex vivo Hormones/cAMP Production by Leydig Cells

As it was expected, androgenization for 2 months with testosterone significantly decreased LH levels in circulation (Fig. 3A) and increased levels of circulating androgens (Fig. 3B).

Steroidogenic activity of purified Leydig cells obtained from rats after 2-month in vivo treatment with testosterone was significantly impaired testosterone by both doses (Fig. 3). Progesterone productions remained unchanged (Fig. 3C), whereas production of androgens (Fig. 3D) and cAMP (Fig. 3F) dramatically declined. Oppositely and differently from 2-week testosterone treatment, elevated estradiol production was observed in Leydig cells from androgenized rats (Fig. 3E).

In order to monitor steroidogenic capacity of Leydig cells obtained from control as well androgenized rats, we examined progesterone and androgen production in basal and presence of hCG ex vivo. Response of Leydig cells from testosterone-treated rats to dose-dependent hCG-stimulation
in term of androgen production was dramatically reduced (Fig. 3I), whereas progesterone production remained unchanged (Fig. 3G). Activity of steroidogenic enzymes in Leydig cells obtained from control and rats androgenized for 2 months was estimated by ability of Leydig cells to convert different steroidogenic substrates to progesterone and androgens. Conversion of pregnenolone to progesterone which reflects 3βHSD activity was significantly, dose-dependently, stimulated in Leydig cells from androgenized rats (Fig. 3H), whereas androgen production dramatically decreased (Fig. 3J). The conversion rate of progesterone to testosterone dramatically declined in Leydig cells from androgenized rats. In the presence of saturating dose of progesterone, conversion rate was reduced about 66% with low dose of testosterone and about 70.6% with 5× higher dose of testosterone (Fig. 3K). The activity of 17βHSD measured by conversion of Δ4-androstenedione to testosterone dose-dependently declined in Leydig cells from rats treated with testosterone for 2 months and effect was potentiated with higher dose (Fig. 3L).

All together, results from the in vivo and ex vivo secretion analysis showed that both doses of testosterone-impaired Leydig cell steroidogenic capacity and activity. To define causation and background of this, we run the gene/protein expression analysis.

**Testosterone Treatment for 2 Months Disturbed the Expression of Steroidogenic Machinery Elements in Leydig Cells**

Expression of specific components of steroidogenic machinery was analyzed by extracting mRNA from purified Leydig cells obtained from controls and androgenized male rats. Gene and protein expression levels were accessed by RQ-PCR and Western blot, respectively.
RQ-PCR analysis revealed that 2-month-androgenization significantly inhibited transcription of Lhr, Scarb1, Tspo, Star, and CYP enzymes (Cyp11a1 and Cyp17a1) involved in androgenesis (Fig. 4A). Oppositely, the expression of Hsd3b, Cyp19a1, Ar, and Pde4a4b (Fig. 4A) was stimulated. Surprisingly and same as after 2-week treatment, the expression of Sf1, Hsd17b, Shbg, and Esr1 was not affected (Fig. 4A).

Results of Western blot analysis were in line with the gene expression and confirmed decreased level of StAR protein but increased protein level for 3βHSD and AR in Leydig cells from rats androgenized for 2 months, independently of applied testosterone dose (Fig. 4B).

Because in vivo studies using two doses of testosterone (0.5 mg/100 g body weight or 2.5 mg/100 g body weight) and three time points (T-1 day, T-2 weeks, and T-2 months) showed, as a most striking upregulated AR and 3βHSD in testosterone-impaired Leydig cells steroidogenesis, we perform in vitro experiments.

The Expression of Steroidogenic Machinery Elements Was Disturbed After Direct In Vitro Treatment With Testosterone

To evaluate the direct influence of testosterone treatment on Leydig cell steroidogenic machinery elements, in vitro experiments were performed. The primary culture of Leydig cells isolated from intact rats was incubated in absence/presence of increasing doses of testosterone (0.1, 1, 10, and 100nM and 1, 10, and 20μM). Our results clearly showed that adding testosterone to the culture medium gradually increased Hsd3b gene expression (Fig. 5) which is in line with effect of in vivo treatment with testosterone. Also in line with all in vivo treatment was gradually decreased expression of Cyp11a1 and Cyp17a, whereas decreased Cyp19a1 expression is in line only with 2 weeks of in vivo testosterone treatment (Fig. 5). Finally, in line with all in vivo treatments, in vitro testosterone addition to the culture medium did not change the expression of Hsd17b and Shbg (Fig. 5). Contrary to in vivo testosterone paradigm, the expression of Ar, Scarb1, Tspo, and Star was not changed with any investigated testosterone concentration, whereas unchanged Lhr level is in line only with T-2 weeks treatment (Fig. 5). In addition and differently from in vivo effects of testosterone is gradually decreased Sf1 and Esr1 expression following in vitro treatment with higher testosterone concentrations (Fig. 5), whereas level of transcripts for both genes remained unchanged after all types of in vivo treatments (Figs. 2 and 4).

DISCUSSION

In this study, we systematically analyzed steroidogenic machinery in Leydig cells isolated from rats treated with high doses of testosterone-enanthate a wide used and abused AAS. The Leydig cells were used to examine ex vivo secretion of steroid hormones, as well as the expression of the genes for the proteins involved (LHR, TSP0, StAR, CYP11A1, 3βHSD, CYP17A1, 17βHSD, and CYP19A1) and related (SCARB1, PDE4A, and PDE4B) to steroid biosynthesis, as well as steroid function (SHBG, AR, and ESR1).

Our investigations reported that prolonged treatment of intact rats with testosterone significantly disturbed steroidogenic machinery and remarkably reduced the capacity of purified Leydig cells to produce testosterone ex vivo. Several lines of evidence proved this. First, the level of AR protein increased in Leydig cells from rats androgenized for 2-week and 2-month treatment with testosterone-enanthate. Second, the expressions of all components of steroidogenic machinery in Leydig cells, except Hsd17b were affected with prolonged androgenization. The expression of Star, Cyp11a1, and Cyp17a1 was inhibited, whereas only transcription of Hsd3b was stimulated. Levels of Cyp19a1 transcript decreased after 2 weeks but increased after 2-month treatment. The conversions of pregnenolone to progesterone increased, whereas the transformation of progesterone to androgens decreased. Third, the level of transcript for PDE4, enzyme exclusively responsible for cAMP degradation, increased. Finally, as a consequence, basal and hCG-supported progesterone, androgen, and estrogen production by Leydig cells obtained from androgenized rat were impaired. In addition, direct in vitro testosterone application also decreased Cyp11a1, Cyp17a1, and Cyp19a1 expression, whereas only transcription of Hsd3b was stimulated.

In this study, we have shown that testosterone, significantly increased the expression of AR gene/protein in Leydig cells of adult rats. This is in line with the studies demonstrated that the mRNA levels for LHR, AR, and 3 alpha-hydroxysteroid dehydrogenase were significantly increased by exogenous in vivo testosterone application (30 μg/day) to NalGlu-treated rats during days 14–21 postpartum (Shan et al., 1995). On the other hand, in utero exposure (from gestation days 12–19) to various AR antagonists (flutamide, linuron, vinclozolin, and p,p'-DDE) didn’t alter the pattern or expression level of AR, and the testosterone levels were unchanged in the fetal testes in any treatment group (Mu et al., 2006). In line with our results are findings that androgens upregulated AR expression in several cell types of human skeletal muscle in vivo and in vitro (Sinha-Hikim et al., 2004). It has been demonstrated the cell-specific and divergent regulation of AR-mRNA turnover by androgen as well as androgen-induced posttranscriptional regulation of AR-mRNA (Bhasin, 2010; Bhasin et al., 2006). The lack of functional AR in Leydig cells has a major influence on Leydig cells steroidogenic functions, as well as spermatogenesis and fertility (Wang et al., 2009). It has been shown that AR signaling in Leydig cells displays autocrine regulation (Hales et al., 1987). In addition, in mice with a unique hypomorphic AR mutation, which disrupts the feedback loop governing testosterone synthesis, genes involved in cholesterol biosynthesis/uptake and steroid biosynthesis are upregulated (Eacker et al., 2008).
We further defined changes at each step of Leydig cells steroidogenesis. It is well established that LH-LHR-cAMP signaling is a key regulator of male fertility through its effects on testosterone secretion, but, transcriptional control of this is poorly understood (Dufau, 1998; Zhang and Dufau, 2003). Our results showed significant decline in the level of LH in serum 24 h after the first testosterone injection, staying declined after 2 weeks and with dramatic decrease after 2 months. This observation is also in agreement with literature (Anderson and Wu, 1996). Our results of transcriptional analysis showed that only 2-month testosterone treatment decreased the transcript for LHR.

In this study, we showed the dramatic decrease of cAMP in Leydig cells from rats androgenized for 2 months. This observation is most probably consequence of dramatic decline of circulating LH accompanied with decreased LHR expression and increased level of PDE4 transcript in Leydig cells from androgenized rats. Declined cAMP level could be the one of the reasons for observed decrease in expression of Star, Cyp11a1, and Cyp17a1, known targets of cAMP-stimulated transcription.

In Leydig cells, cAMP-regulated Star promoter activity primarily involves SF1, CREB/CREM, and GATA4 and is modulated by other transcriptional cofactors (Lavoie and King, 2009). The orphan nuclear receptor and transcription factor SF1 (now termed NR5A1) is expressed in all steroidogenic tissues. SF1 has been shown to increase expression of the steroidogenic machinery by binding to its response element site found in the promoter regions of the genes encoding for STAR, CYPs, and 3βHSD (Martinez-Arguelles and Papadopoulos, 2010). Although our results demonstrated that prolonged androgenization decreased expression of STAR transcript/protein, we were not able to detect changes in SF1 expression. The lack of a effect of testosterone-estriathane on SF1 expression in purified Leydig cells suggest that other transcription factors/signaling-pathways, alone with cAMP CREB/CREM, are more affected with androgenization. In addition, recent study showed that the steroidogenesis in Leydig cells could be disturbed by androgen receptor corepressor-19kD (ARR19), an anti-steroidogenic factor negatively regulated by LH-cAMP signaling via the control of GATA-1 (Qamar et al., 2009).

In this study, we showed that the most sensitive components of steroidogenic machinery in Leydig cells are the key CYP enzymes, Cyp11a1 and Cyp17a1. Only the expression of these transcripts was inhibited 24 h after the testosterone injection. CYP11A1 is the first enzyme controlling the synthesis of all steroid hormones. Basal and/or cAMP-mediated gonadal regulation of the Cyp11a1 involves SF1/LRH-1, Sp1, GATA4, CREB-1, and AP-1 family members. In Leydig cells, Cyp11a expression increases more slowly than StAR in response to LH/hCG-LHR-cAMP signaling (Lavoie and King, 2009). Down-regulation of LH/hCG-LHR-cAMP signaling in Leydig cells following 2-month testosterone treatment could be the cause of declined Cyp11a1 expression. The expression of Cyp17a1 is regulated by a variety of tissue-specific and species-specific transcription factors (Shi et al., 2009). Our results obtained in vivo and in vitro extend previous work reported the sensitivity of Cyp17a1 to androgen treatment in vitro. It has been shown that testosterone represses cAMP-stimulated Cyp17a1 mRNA in both primary Leydig cell cultures (Hales et al., 1987) and in MA-10 Leydig tumor cells (Burgos-Trinidad et al., 1997). It was reported that testosterone produced during cAMP induction of CYP17A1, negatively regulates the amount of this cytochrome P450 enzyme by two distinct mechanisms: by repressing an AR-mediated-cAMP-induced synthesis of CYP17A1 and by increasing the rate of CYP17A1 degradation (Hales et al., 1987). In addition, AR-mediated repression involves binding of the AR to sequences in the cAMP-responsive region of the Cyp17 promoter, possibly interfering with the binding of the protein(s) that mediate cAMP induction of Cyp17 (Burgos-Trinidad et al., 1997). Accordingly, we can speculate that decreased Cyp17a1 is consequence of strong upregulation of AR gene/protein, together with downregulation of LH/hCG-LHR-cAMP signaling in Leydig cells from androgenized rats.

Results of this study showed, for the first time, that prolonged treatment with high doses of testosterone significantly increased 3βHSD gene/protein levels, and as consequence, progesterone production, suggesting that testosterone may upregulate progesterone synthesis. It is interesting that the 3βHSD is the only steroidogenic enzyme upregulated in Leydig cells obtained from androgenized rats, as well as in Leydig cells treated in vitro with testosterone. More conclusive evidence for this hypothesis we obtained in experiments with pregnenolone, substrate for 3βHSD. Despite decreased expression of upstream components of steroidogenic machinery (Star and Cyp11a1) following the testosterone treatment, increased 3βHSD expression and enzymatic activity could prevent the complete block of testicular steroidogenesis. Studies in cultured rat Leydig cells demonstrated that activation of LH-LHR-cAMP signaling increased 3βHSD mRNA/protein/activity after 24–72 h of treatment (Payne and Hales, 2004). Because our results showed downregulation of LH-LHR-cAMP signaling following testosterone treatment, we proposed AR-dependent mechanism for upregulation of 3βHSD expression. Furthermore, the expression pattern of 3βHSD at two time points, 14 and 60 days of testosterone application, could be correlated with increased AR expression. It is know that regulation of 3βHSD expression involve multiple signal transduction pathways, initiated by several growth factors, steroids, cytokines, and ending with activation of transcriptional factors, including STAT5a/b, responsible for the fine control of 3βHSD gene (Lavoie and King, 2009; Simard et al., 2005). The functional cooperation and link between STAT5a/b and AR have been shown (Kloever et al., 2009; Tan et al., 2008). Thus, our results showing the upregulation of 3βHSD expression following both, in vivo and in vitro, testosterone treatments are in line with this.
It is noteworthy that testosterone treatment didn’t affect the expression of Hsd17b, the last and key enzyme for testosterone formation. In the same time, the activity of 17βHSD measured by conversion of Δ4-androstenedione to testosterone dose-dependently declined in Leydig cells from rats treated with testosterone for 2 weeks and 2 months. The possible explanation for this could be the either increased degradation of 17βHSD protein or/and affected activity of this enzyme.

The results of the present study also showed that androgenization for 2 weeks inhibited Cyp19a1 expression and estradiol production, whereas 2-month treatment stimulated both, the expression of Cyp19a1 and estradiol production. The increase in estradiol production could have the potential side effects in males, such as beneficial effects on skeletal growth and bone maturation, may be protective against Alzheimer’s disease, beneficial effects on the cardiovascular system. However, the potentially adverse effects of estrogens on the prostate occurs typically in old age, when there is usually a shift in the estrogen/androgen ratio in favor of estradiol production.

Finally, our observations suggesting that androgenization strongly affects steroidogenesis, at least in part through AR-dependent mechanisms are in agreement with a recent report showing that a unique AR mutation caused opposite effect on steroidogenic genes (Eacker et al., 2008). Thus, upregulation of AR and 3βHSD in testosterone-impaired Leydig cells steroidogenesis could be the possible mechanism that maintain and prevent loss of steroidogenic function. In addition, here, we provided numerous data substantiating the hypothesis that application of testosterone-enanthate, widely used and abused AAS, dramatically disturbs steroid-homeostasis, which questioning the clinical use, in sharp contrast to the potential health risks.

SUPPLEMENTARY DATA
Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING
Serbian Ministry of Science and Technological Development (173057) and Autonomic Province of Vojvodina (0253).

ACKNOWLEDGMENTS
We are very grateful to Prof Ian Masson (University of Edinburgh) for very kind donation of 3βHSD antibody, Prof Douglas Stocco (Texas Tech University) and Prof Dale Hales (University of Illinois) for generous and continuous donation of StAR antiserums. We appreciate Prof Gordon Niswender (Colorado State University) for supplying antibodies for RIA analysis.

REFERENCES


